QUALITY ASSURANCE PROJECT PLAN

eDNA MONITORING OF BIGHEAD AND SILVER CARPS

Prepared for:

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REVIEW CERTIFICATION SHEETS
FOR

Final Quality Assurance Project Plan
for the
eDNA Monitoring of Bighead and Silver Carp

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TABLE OF CONTENTS

Section No. **Page No.**

1. PROJECT DESCRIPTION AND PERSONNEL REQUIREMENTS 1-1
1.1 Background 1-1
1.2 General Requirement 1-2
1.3 Project History 1-2
1.4 Objective 1-2
1.5 Project Personnel 1-3
1.6 Reporting: 1-5
1.7 Case number assignment and management: 1-6
1.8 QAPP maintenance and modifcations 1-6
1.9 eDNA Security Plan(s) 1-7

2. SAMPLE COLLECTION 2-1
2.1 Pre-Trip Planning and Site Selection 2-1
2.1.1 Purpose 2-1
2.1.2 Pre-trip Planning Procedure 2-1
2.1.3 Field equipment list 2-2
2.1.4 Lotic system Site Selection Procedure (in the field) 2-3
2.1.5 Lentic system Site Selection Procedure (in the field) 2-4
2.1.6 eDNA Trailer Preparation and Field Considerations 2-5
2.2 Equipment Preparation 2-5
2.2.1 Purpose 2-5
2.2.2 Equipment Procedure for Coolers and Sample Bottles 2-6
2.2.3 Boat and Field Equipment 2-7
2.2.4 Boat and Equipment Decontamination Procedure 2-7
2.3 Motorized Sample Collection Procedure 2-9
2.3.1 Purpose 2-9
2.3.2 Water Collection Procedure 2-9
2.4 Wading and Non-motorized Sample Collection Procedure 2-11
2.4.1 Purpose 2-11
2.4.2 Wading Water Collection Procedure 2-11
2.4.3 Non-motorized Water Collection Procedure 2-12

3. SAMPLE PROCESSING (FILTERING or CENTRIFUGING) 3-1
3.1 Purpose 3-1
3.2 Filtering Procedure 3-1
3.2.1 Laboratory Preparation 3-2
3.2.2 Sample Preparation 3-2
3.2.3 Filtering the Equipment Control 3-2
3.2.4 Filtering the Sample 3-3

3.2.5 Equipment and Work Area Cleaning After Filtering Each Sample 3-4
3.3 Centrifuging Procedure 3-5
3.3.1 Laboratory Preparation 3-6
3.3.2 Sample Preparation 3-6
3.3.3 Centrifuging the Samples 3-6

4. SAMPLE SHIPMENT 4-1
4.1 Purpose 4-1
4.2 Shipping Procedure 4-1

5. DNA ASSAYS (TO BE UPDATED IN 2014) 5-1
5.1 General Quality Assurance and Chain-of-Custody Considerations 5-1
5.2 Quality Control for Sample Custodian Procedure and Storage 5-2
5.3 Physical Separation of Pre-PCR and Post-PCR Assay Stages 5-3
5.3.1 The eDNA Extraction Room 5-3
5.3.2 Pre-PCR Room 5-3
5.3.3 PCR Product Analysis Room 5-3
5.4 Receipt of Filters/Tubes 5-3
5.4.1 Source 5-3
5.5 DNA Extraction from Filters or Tubes 5-4
5.5.1 Source 5-4
5.5.2 DNA Extraction Quality Assurance and Chain-of-custody 5-4
5.5.4 PowerWater Procedure for *filters* 5-6
5.5.5 Qiagen DNeasy Kit (or equivalent kit) Procedure for *filters* 5-8
5.6 PCR Amplification of eDNA Samples 5-10
5.6.1 Purpose 5-10
5.6.2 Source 5-10
5.6.3 PCR Quality Assurance and Chain-of-custody 5-10
5.6.4 Procedure 5-11
5.7 Gel Electrophoresis of eDNA PCR Assays 5-13
5.7.1 Purpose 5-13
5.7.2 Source 5-13
5.7.3 Gel Electrophoresis Assurance and Chain-of-custody 5-13
5.7.4 Option A Procedure 5-14
5.7.5 Option B Procedure 5-14
5.8 eDNA Gel Documentation and Storage 5-15
5.8.1 Purpose 5-15
5.8.2 Source 5-15
5.8.3 Gel Documentation and Storage Assurance and Chain-of-custody 5-15
5.8.4 Procedure 5-16
5.9 Gel Interpretation 5-16
5.9.1 Purpose 5-16
5.9.2 Source 5-16
5.9.3 Gel Interpretation Quality Assurance and Chain-of-custody 5-16

5.10 Confirmation of Positive Results 5-17
5.10.1 Purpose 5-17
5.10.2 Source 5-17
5.10.3 Gel Interpretation Assurance and Chain-of-custody 5-17
5.10.4 Procedure 5-17
5.11 Communication of eDNA Assay Results 5-21
5.11.1 Purpose 5-21
5.11.2 Source 5-21
5.11.3 Quality Control 5-21
5.11.4 General Procedure (for details see Appendix E for SOP) 5-21

6. INTERNAL QUALITY CONTROL CHECKS 6-1
6.1 Laboratory Quality Control Evaluation Criteria 6-1

7. SPECIFIC ROUTINE PROCEDURES TO ASSESS DATA PRECISION, ACCURACY, AND COMPLETENESS 7-1
7.1 Field Methods and Laboratory Data 7-1

8. CORRECTIVE ACTIONS 8-1

9. PREVENTATIVE MAINTENANCE PROCEDURES 9-1
9.1 Field Equipment/Instruments 9-1
9.2 Laboratory Instruments 9-1

10. PERFORMANCE AND SYSTEM AUDITS 10-1
10.1 Field Audits 10-1
10.2 Laboratory Audits 10-1

LIST OF TABLES

Table 5-1. List of critical components for eDNA Processing, and component information for lab work. No vendor is listed for generally available supplies 5-22

LIST OF EXHIBITS

1	Field Collection Summary	11-2
2	Field Sample Datasheet	11-4
3	Chain-of-Custody (COC) Record	11-5
4	Sample Receipt Checklist, internal lab COC	11-6
5	Excel Log File for Lab Cases	11-7
6	Excel Log File of a Single Case	11-8
7	-20 Freezer Temperature Log	11-9
8	Cooler Storage Temperature Log	11-10
9	-80 Freezer Temperature Log and Sample Log	11-11
10	Ambient Storage Sample Log	11-12
11	PCR Log	11-13
12-14	Lab Data Sheets	11-14
15	QAPP Compliance Certification Statement	11-19
16	Quality Control Audit Checklists for Field Sampling and Processing	11-20

LIST OF APPENDICES

A	Roles and Responsibilities and Annual Staff Assignments
B	Results Communication SOP (TO BE UPDATED IN 2014)
C	Data Management and Database Instruction Manual
D	Sample Bottle Management Plan
E	Methods for Elimination and Reduction of eDNA on Boats and Equipment
F	Mobile eDNA Trailer Maintenance and Use Manual
G	eDNA Security Plan for Co-Located La Crosse FWCO and WGL

ACRONYMS/ABBREVIATIONS

COC	Chain-of-custody
CSO	combined sewer overflow
DI	deionized (water)
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates, also known simply as nucleotides
eDNA	environmental Deoxyribonucleic acid
FWCO	Fish and Wildlife Conservation Office
GL	Great Lakes
GPS	Global Positioning System
LDB	left descending bank
MRWG	Monitoring and Response Work Group
MSDS	Material Safety Data Sheets
PCR	polymerase chain reaction
QAPP	Quality Assurance Project Plan
RDB	right descending bank
Taq	*Thermus aquaticus*
USACE	United States Army Corps of Engineers
USFWS	United States Fish and Wildlife Service
UV	Ultraviolet light
WGL	Whitney Genetics Lab

SECTION 1

1. PROJECT DESCRIPTION AND PERSONNEL REQUIREMENTS

1.1 Background

Invasive aquatic nuisance species pose a major threat to aquatic ecosystems worldwide. Within Illinois, the manmade Chicago Sanitary & Ship Canal (CSSC), constructed in the early 1900s, provided an unnatural portal for invasive species dispersal between the geologically separated Mississippi River and Great Lakes drainage basins. In 2002, in an effort to curtail the spread of invasive species between the two basins, the U.S. Army Corps of Engineers (USACE), constructed a dispersal barrier system within the CSSC. The primary objective of the barrier system when initiated was to stop the dispersal of the invasive Round Goby into the Mississippi River basin; however, once the project was completed, it was found that the Round Goby had already surpassed the barrier. Since then, a new threat to the Great Lakes from the Mississippi River basin has become the primary objective of the dispersal barrier system. Invasive Asian carps, including Bighead Carp (*Hypophthalmichthys nobilis*) and Silver Carp (*H. molitrix*) have been steadily dispersing upstream through the Mississippi, Illinois, and Des Plaines rivers. Their potential dispersal through the dispersal barrier system within the CSSC poses a potential threat to the Great Lakes ecosystem.

In the past, traditional fishery techniques were used to detect the leading edge of the Asian carp population; however, this method was somewhat ineffective at targeting these species at low densities. The University of Notre Dame, with funding from the USACE, developed a method that detected "environmental" DNA (eDNA) left behind in the aquatic system by the targeted species. Environmental DNA enters the system through a variety of mechanisms, some of which include sloughing of external epidermal cells into the water, sloughing of internal epidermal cells into feces and into the water, and as tissue residues following injury or predation. The detection of eDNA in water samples is based on whole DNA extraction from particulate organic and inorganic matter found in the water and polymerase chain reaction (PCR) assays for species-specific mitochondrial DNA markers.

Use of this method is to provide detection of Asian carp at low densities and to serve as an early detection system of the spread of Asian carp into previously uninhabited areas. The original Quality Assurance Project Plan (QAPP) detailing the eDNA monitoring process, including methodologies and quality controls, was requested from the U.S. Army Engineer Research and Development Center (ERDC) as the USACE assumed eDNA monitoring responsibility from the University of Notre Dame. This modified QAPP will be followed by the U.S. Fish and Wildlife Service (USFWS) offices responsible for field sampling and lab processing of eDNA samples. Fish and Wildlife Conservation offices (FWCO) will handle field sampling and the Whitney Genetics Lab (WGL) at the La Crosse Fish Health Center will process eDNA samples since the USFWS assumed responsibility for monitoring Asian carp in the Chicago Area Waterway System (CAWS) and other water bodies in 2013.

This version of the QAPP has been modified to be a general guide to field and laboratory methods that should be employed during eDNA monitoring programs. Specific sampling plans and schedules for particular water bodies will be developed by regional sampling agencies and their partners, as well as overseeing governing bodies (such as the Asian Carp Regional Coordinating Committee and Great Lakes Commission). These plans are available online on various agency websites and will not be included in this document.

1.2 General Requirement

USFWS Midwest Region requires a Quality Assurance Project Plan (QAPP) for eDNA monitoring. Full-scale eDNA monitoring commenced in September 2010 by the USACE, and based on consultation with experts in processing of litigable DNA evidence (which applies to eDNA monitoring), a final, comprehensive QAPP was not in place within that time frame. Instead a provisional QAPP was used during the first year of eDNA monitoring, to be followed later by a finalized version under a different scope of work. The USFWS will continue to maintain and update a QAPP for use in eDNA monitoring programs that allows for inclusion of any beneficial technical or strategic modifications that become apparent from past monitoring events, research conducted by the eDNA calibration research team, or research published in the literature and vetted by the eDNA calibration team or other USFWS genetics laboratories.

1.3 Project History

The University of Notre Dame, Department of Biological Sciences, Center for Aquatic Conservation, prepared a Standard Operating Procedure (SOP) in 2010. The SOP provided details regarding eDNA monitoring protocol and was given to USACE in May 2010 in compliance with Cooperative Ecosystem Study Unit agreement #W912HZ-08-2-0014, modification P00007. On 15 and 16 December 2009, a technical and quality systems audit of the Center for Aquatic Conservation Lodge Laboratory at the University of Notre Dame was conducted by the US Environmental Protection Agency. The laboratory audit report dated 5 February 2010 was provided to USACE in addition to the eDNA monitoring protocol. These documents served as the basis for the QAPP followed by ERDC. Additionally, USACE submitted the eDNA methodology for an Independent External Peer Review (IEPR), which is a requirement to examine decision documents and supporting work products where there are public safety concerns, significant controversy, a high level of complexity, or significant economic, environmental and social effects to the nation. Released fall of 2011, the eDNA IEPR report, conducted by objective panelists with technical expertise in genetics and population ecology, confirmed eDNA sampling and testing methodology is sound for detecting Silver and Bighead carp DNA but cannot indicate the source of Asian carp DNA (information on the size, gender, age and number of individuals present and cannot distinguish between pure Silver or Bighead carp and their hybrids). In 2013, the WGL received the QAPP and made changes so that it would be applicable to the new field sample processing and eDNA processing personnel and their specific locations. It was also modified according to results of the eDNA Calibration Studies (ECALS) and lab validations carried out by the interagency ECALS team. Annual modifications (see section 1.8) to the QAPP will be approved by regional field staff, the USFWS eDNA team, and leadership of USFWS Midwest Region. Changes to sample analysis will undergo peer-review by external experts and validation in specifically designed studies carried out in at least three different laboratories.

1.4 Objective

The objective of this QAPP is to provide detailed procedures for Asian carp eDNA sample collection, sample processing (including filtering, centrifuging, DNA extraction, PCR, biomarker analysis, DNA sequencing), data reporting, and quality control/quality assurance procedures to ensure that data are as technically defensible, consistent, and usable as possible. The specific goals and objectives of sampling plans for particular bodies of water are currently directed by the appropriate governing body. For example, the Chicago Area Waterway sampling plan has been issued by the Asian Carp Regional Coordinating Committee's Monitoring and Response Workgroup in the Monitoring and Response Plan. The Great Lakes, Ohio River, and Upper Mississippi River plans will be or have been issued by USFWS Midwest Region in conjuction with state and other partners.

1.5 Project Personnel

The eDNA monitoring project in USFWS Midwest Region has formed an eTeam, with members of the Regional Office, all field stations and the Whitney Genetics Lab. An official document defining roles and responsibilities is attached in Appendix A, and the names and contact numbers should be updated annually.

The eDNA monitoring program of USFWS Midwest Region must have personnel appointed to the following positions, and it is highly recommended for other agencies interested in eDNA monitoring:

Field sampling point of contact for each field station responsible for sampling and field processing. For each sampling trip, specific personnel will differ for each role as listed in the QAPP, but each role will be filled on each sampling trip

- eDNA Project Coordinator
- Sampling Leader
- Sampling Quality Assurance Specialist
- Sample Processing Leader
- Sample Processing Quality Assurance Specialist
- DNA Processing Leader
- DNA Processing Quality Assurance Specialist
- Data Documentation & Reporting Specialist
- Supporting Agency contacts

The minimal responsibilities of the above positions are detailed below. Specific personnel assigned to the project are listed in Appendix A.

Sampling and Sample Processing roles may NOT be filled by the same person and must each be filled on every sampling trip.

Prior to each trip, the eDNA Program Coordinator and the eDNA Processing Leader need the name and mobile phone number for the field sampling and sample processing leaders. After each trip, a brief summary report (Exhibit 16) covering quality assurance issues and any changes in personnel roles should be provided to the eDNA Program Coordinator.

eDNA Project Coordinator: USFWS Midwest Regional Office staff specialist responsible for development, coordination and implementation of the USFWS eDNA monitoring program for Asian carps. Assumes principal responsibilities for initiating, leading, facilitating, integrating, coordinating, and communicating necessary monitoring work and activities using eDNA of the Midwest Region's Fisheries Program through the cooperative conservation community. Provides guidance for implementing and utilizing eDNA activities at the field level. Provides technical leadership to plan, conduct and lead other biologists as well as coordinate activities to identify and coordinate surveillance areas in water bodies for the early detection of Asian carp eDNA. Ensures the best scientific practices are used during the development and implementation of management plans to monitor, control, and eradicate Asian carp. Implements and coordinates the eDNA collection program; eDNA processing and interpretation of data; collection and dissemination of research information from institutions and research agencies on findings and new developments in eDNA collection and interpretation of data and the development and implementation of Asian carp management plans. Responsible for the overall QAPP and its implementation at the field and lab levels. Responsible for communication between the lab and Regional office. Integration with interagency ECALS team and other relevant entities.

Sampling Leader: Responsible for obtaining water samples for eDNA monitoring and providing those to sample processing team. Responsible for reporting results to Sample Processing and DNA Processing Team Leaders, the eDNA Program Coordinator, as well as other designated USFWS personnel.

Sampling Quality Assurance Specialist: Responsible for knowing all quality assurance/quality control (QA/QC) measures for eDNA sampling efforts. Advises Sampling Leader on any potential QA/QC problems. Reviews procedures, field logs, data collection methodology; ensures that all agencies participating in sampling are conforming to procedures, and documents this after each sampling trip (Exhibit 16). Recommends corrective actions for non-conformities.

Sample Processing Leader: Responsible for filtering/centrifuging water samples for eDNA monitoring and providing those processed samples to eDNA Processing team. Responsible for reporting results to Sampling and DNA Processing Team Leaders, the eDNA Program Coordinator, as well as other designated USFWS personnel.

Sample Processing Quality Assurance Specialist: Responsible for knowing all QA/QC measures for filtering/centrifuging efforts. Advises Sample Processing Leader on any potential QA/QC problems. Reviews procedures, laboratory logs, and documentation for processing; ensures all personnel are conforming to procedures, and documents this after each sampling trip (Exhibit 16). Recommends corrective actions for non-conformities.

DNA Processing Leader: Responsible for processing eDNA samples through DNA extraction, PCR, and sequencing. Responsible for reporting results to eDNA Program Coordinator, as well as other designated USFWS personnel.

DNA Processing Quality Assurance Specialist: Responsible for knowing all QA/QC measures for eDNA processing efforts. Advises eDNA Processing Leader on any potential QA/QC problems. Reviews procedures, laboratory logs, and documentation for DNA processing; ensures all personnel are conforming to procedures. Recommends corrective actions for non-conformities.

Data Documentation & Reporting Specialist: Assists DNA Processing Leader in maintaining laboratory database for eDNA sample processing. Performs data completeness and data verification checks, and ensures that all data are documented completely.

Assigned Project Leaders and Specialists: Others serving on the project may include researchers, technicians, and budgetary personnel. Sampling may employ personnel from other agencies in coordinated efforts. All personnel must meet a minimum standard for training and/or experience before independently conducting any portion of the eDNA monitoring protocol. Each station's lead eDNA personnel will attend an annual QAPP review training (train the trainer). These lead eDNA staff will then train any other staff assisting with eDNA work. The supporting agency contacts are given in Appendix A. Minimum personnel training requirements are given below.

Personnel Training Requirements

Minimum training and/or experience requirements for the different major components of the eDNA monitoring protocol are detailed below.

Boat Operator:

- Must meet USFWS boat operator requirements as a minimum.

Sampling:

- A BA/BS degree or its equivalent in biology or related field of study, or
- At least 2 years of specialized postsecondary training or an associate degree in applied science or science-related technology, or
- A high school diploma or its equivalent and a minimum of 2 years professional experience in biology-related field.
- First aid and/or boating safety course.
- Minimum 1 year experience in collecting field samples for biological analyses.

Sample Processing:

- A BA/BS degree or its equivalent in biology or related field of study, or
- At least 2 years of specialized postsecondary training or an associate degree in applied science or science-related technology, or
- A high school diploma or its equivalent and a minimum of 2 years professional experience in biology-related field.
- First aid training.
- Facility-specific safety training.
- Minimum one semester college level laboratory experience, plus eDNA-specific training.

DNA Processing:

- A minimum BA/BS degree or its equivalent in biology or related area and successful completion of college course work (graduate or undergraduate level) covering the subject areas of biochemistry, genetics, and molecular biology (molecular genetics, recombinant DNA technology) or other subjects that provide a basic understanding of the foundation of DNA analysis, as well as course work and/or training in PCR amplification as it applies to eDNA analysis.
- A minimum of 6 months of general DNA laboratory experience, including experience with DNA extraction and PCR. Additionally, 2 weeks of training on Asian carp eDNA protocols.
- Successful completion of a qualifying test demonstrating effective execution of eDNA-type assays before beginning independent work on the project.

1.6 Reporting:

- All agencies, need to submit resumes (Curriculum Vitaes) for proposed staff to the eDNA Program Coordinator, to be reviewed and approved by the eDNA Program Coordinator. As new personnel are acquired and trained, their resumes must be filed with the eDNA Program Coordinator. Those documents will be kept with the project file. This paperwork should be updated each time the QAPP is updated.
- For each sampling event, a pre-trip summary of the sampling sites, dates, personnel roles, and contact information should be supplied to the eDNA Program Coordinator, other USFWS personnel as needed, and partner agencies as appropriate.
- Specific staff member names conducting the various activities must be documented on all official data sheets (e.g., on the field sampling log).
- Pre-trip communications with eDNA Program Coordinator may be via email or text.
- Upon completion of all sampling events, the trip completion report (Exhibit 16) must be filled out and filed with the eDNA Program Coordinator. This form includes areas to clearly list case

numbers, sample numbers, sampling locations, personnel, and ensures that the Sampling and Sample Processing Quality Assurance Leads confirm adherence to the QAPP.

- The lab will file weekly reports with the eDNA Program Coordinator that contain up-to-date information on the progress of samples within each case. The lab will file weekly reports on general case progress without specific results with the FWCO Project Leaders so they are kept up-to-date on lab progress.
- The lab will update the eDNA Program Coordinator with information regarding presumptive positive samples so that timely handling of final results can be mapped and communicated internally and with other offices and partners as needed.
- The lab will file final reports for each case that contains all of the processing information and final positive and negative results with the eDNA Program Coordinator.

1.7 Case number assignment and management:

All eDNA field and lab data will be coallated and housed in a geo-referenced database maintained at the Regional Office. The database is organized around a unique reference ID for each individual sample, thus a unique identifier is required for each eDNA sample.

- Each sampling event is centered around a specific water body, and will be assigned a case number consisting of a 5-digit integer (00000). Sample ID numbers within cases will be in consecutive numerical order, and will consist of a 3-digit integer (000). Each sample will be identified by the 8-digit number consisting of the case and sample ID number, without spaces or punctuation (00000000).
- WGL will create a master case list each season and assign case numbers as needed to each FWCO. FWCOs will assign case numbers to sampling events so that the lab remains blind for sample processing.
- A table of case numbers will be provided by WGL to the eDNA Project Coordinator so that case numbers will never be repeated among sampling years.
- Sampling priority will be finalized by the eDNA Project Coordinator and appropriate FWS and partner personnel.
- Lab processing priority (which may differ from sampling priority) will be finalized by the eDNA Project Coordinator and appropriate USFWS and partner personnel.

1.8 QAPP maintenance and modifcations

Early detection of aquatic species by eDNA is a new technique that is simultaneoulsly undergoing extensive development on the research front as it is applied on the ground in monitoring efforts. This results in rapid advancement of the technique that should be applied to monitoring efforts to not only improve use of the tool in management applications, but also to improve efficiency.

- The USFWS works closely with collaborating agencies such as the USGS and the USACE to keep up with current research so that the eDNA monitoring program can be updated regularly.
- The QAPP should be updated annually to reflect advancements in eDNA research as well as to improve efficiency in field and lab efforts based on lessons learned from the previous year of implementation.
- Annual updates to the QAPP may be implemented by two mechanisms.
    - Changes to field methods and anti-contamination measures by the eTeam. In 2014 USFWS FWCOs, WGL, and the Midwest Regional Office formed an eDNA team (eTeam, see Appendix A) to work on maintaining the QAPP.

        - These changes are suggested by field staff, and reviewed by the eTeam, and upon approval, implemented into the QAPP. In general, these changes are to accommodate a more-widely implemented monitoring program spatially to include a much greater area than the CAWS. It also accommodates implementation by several different FWCO offices, instead of a fairly limited crew used in the CAWS.
        - None of these changes reduce the level or rigorousness of quality control measures so that the QAPP maintains the high level of quality control that was approved by several external reviews.
    - Changes to lab methods as recommended by collaborating research agencies such as the USGS or USACE or lab staff. New techniques and products must be validated through rigorous studies conducted in at least two labs, preferably three labs.
    - Again, these changes incorporate the same level of rigorous quality control as the original QAPP.
- Other agencies that would like to contract with other labs for eDNA monitoring may want to use the QAPP are encouraged to do so. However it is likely that partnering with other labs will lead to procedures that are not covered in the QAPP. In this case, it is up to each agency to decide on their own requirements needed for eDNA sampling and lab processing.
    - The USFWS recommends a mimimum of following closely sections 1-4, 6-10 to ensure rigorous quality control and sample integrity.
    - Use of other labs should be carefully reveiwed by the contracting agency staff and each agency should ensure that labs are validated and can process samples with at least the same level of quality control and in lab situations that minimize contamination in highly sensitive PCR applications.
        - It is likely that other labs will use assays with different PCR markers and even different PCR techniques such as digital PCR. It is up to the management agency contracting the work to ensure that these assays have been rigorously tested and validated for use in sensitive eDNA monitoring programs. At a mimimum, the assay methods should be published or validated in a rigorous, round-robin lab study.

1.9 eDNA Security Plan(s)

(1) A detailed eDNA security plan for the Midwest Fisheries Center has been developed due to the co-location of the La Crosse FWCO and the WGL. Staff in the FWCO often work in Asian carp contaminated waters and conduct field work where Asian Carp are directly handled. Thus, this plan includes detailed procedures for decontaminating field equipment as well as boats, trailers, and trucks used for all field work including eDNA sample collection. It is a stand-alone document included as Appendix G.

(2) A DNA security plan for each field or lab station involved in eDNA sampling or processing should be developed using the Midwest Fisheries Center eDNA security plan as a model, but adapted for each station's unique situation. This plan should be kept on file at the regional office. The documents should be signed by all personnel at the field or lab station and approved by the eDNA Project Coordinator(s).

SECTION 2

2. SAMPLE COLLECTION

Prior to any field sampling work, all field employees must review this QAPP and acknowledge the procedures and processes to be followed for every sample and every event. Field employees will acknowledge their understanding and intent to comply by signing the certification form given as Exhibit 15. Field employees will also review the sampling safety plan (separate station-specific document) and participate in a safety briefing.

Prior to any sample processing or analysis work, all laboratory employees must review this QAPP and acknowledge the procedures and processes to be followed for every sample and every event. Laboratory employees will acknowledge their understanding and intent to comply by signing the certification form given as Exhibit 15. Laboratory employees will also review the laboratory safety plan (separate station-specific document) and participate in a safety briefing.

2.1 Pre-Trip Planning and Site Selection

Refer to the internet for annual monitoring plans issued by the agency responsible for each particular body of water. For example, the CAWS monitoring plan can be found at www.asiancarp.us.

2.1.1 Purpose

Accurate planning of a general collection site is necessary to effectively manage the time of crews collecting samples, as well as to ensure complete and correct sampling procedures are used.

2.1.2 Pre-trip Planning Procedure

Refer to the annual monitoring plans to properly plan for sampling. The sampling agency should use interactive aerial imagery software (i.e., Google Earth) to scope out reaches to be sampled, placement of samples, and unique features that should be targeted during sampling.

(1) Aerial maps should be detailed enough to show unique features (e.g., barge slips, factory, bridge pilings etc.) that can be identified in the field and used as markers for location when sampling. The recommended minimum scale is 1':500',if that is not available, use the best scale possible.

(2) Aerial maps should be marked with sample locations and should ensure spatial coverage and overall representativeness of the sample area.

(3) Target specific areas (backwaters, island side channels, pooled areas, below and around structures, confluence of tributaries) as well as integrating transect plots in the sampling plan.

(4) Print map(s) with detailed sample plan.

- Locate access points for boat launch and acquire permission to use if necessary. If sampling around locks, or if sampling will require lockage, notify the Lockmaster at least 1 day before sampling.

(5) Coordinate sample plan with sampling crew, eDNA Program Coordinator, or any partner agency.

- Sampling crew should comprise three people at a minimum: one boat operator, one lead sampler, and one sampling assistant.

- Processing crews should consist of two people. All crew members must have their resume (CV) on file with the USFWS (section 1.6), prior to the sampling event.
- All participants must have read this QAPP and must have a signed certification statement (Exhibit 15) on file with the USFWS, prior to the sampling event. All participants involved in the sampling must meet the minimum qualifications given for their role in Section 1.3 of this document.
- A field equipment checklist and datasheets (Exhibits 1&2) should be printed prior to each sampling trip on Rite-in-the-Rain® paper or other waterproof paper. Datasheets may be printed on front and back.
- Check river stage and weather forecast.
- File pre-trip plan with sampling sites, launches, personnel in designated QAPP roles, and field contact information with eDNA Project Coordinator and other appropriate FWS and partner contacts. This can be done via email or text.
- Prior to each trip, the eDNA Program Coordinator and the eDNA Processing Leader need the name and mobile phone number for the field sampling and sample processing leaders

(6) No eDNA sampling should occur within 5 days after a significant rainfall event (more than 1.5 inches in a 24-hr period) on the rising limb of a hydrograph of the river as it exceeds flood stage. In the CAWS, sampling should be avoidedwithin 2 days of a combined sewer overflow (CSO) event. Weather data and river stage for the area to be sampled can be checked at: http://waterdata.usgs.gov/nwis. The occurrence of a CSO event can be verified by contacting the FWCO contact for that particular sampling event listed in Appendix A.

In other waterbodies, sampling during the rising hydrograph may increase the chances of encountering gametes shed during spawning activity, but care should be taken to only sample in safe conditions.

2.1.3 **Field equipment list** (procedures are in section 2.2; to be provided by sampling office/agency

(1) Waterbody-appropriate vessel. If possible, vessel and associated equipment should be dedicated to eDNA collections to minimize risk of DNA contamination of samples from other sources. If boat dedication is not possible, or eDNA collection occurred in known carp positive waters, procedures for decontaminating boats and equipment are included in Section 2.2.4. These procedures will also work for converting a previous carp sampling boat to a dedicated eDNA sample collection vessel.

(2) eDNA-only dedicated personal flotation devices (PFD) for each crew member, using the type of devices listed in the safety plan. Minimum PFD requirements are Type I within the Safety Zone near the existing electric barrier (on the Chicago Sanitary and Ship Canal) or Type II for other areas. NOTE: In some cases, eDNA sampling may occur in Asian carp-infested waters, avoid using clean eDNA dedicated field gear for these trips to prevent contamination for later use in non-infested waters.

(3) If filtering: 100-qt coolers (each cooler capable of holding a minimum of 20 2L samples); decontaminated.

(4) If centrifuging: enough 100-qt coolers capable of holding 5 50-ml centrifuge tubes per planned field sample.

(5) If filtering: 2L sample bottles, autoclavable polypropylene, labeled by sampling agency. New bottles can be used directly out of the box, but used bottles should be decontaminated according

to the section 2.2.2. For specific bottle-handling procedures, see Appendix D. Note: all field offices sending samples to WGL must use bottles prepared for re-use at WGL.

(6) If centrifuging: Sterile, chemical-free polypropylene 50-ml centrifuge tubes, that are rated to withstand 6000 x g, labeled by sampling agency. Tubes will not be re-used, but WGL will return shipping containers to be re-used by sampling agencies if requested.

(7) 3-gallon sprayer (low pressure hand sprayer for spraying down boats in the field), hot water pressure sprayer (for decontaminating boats where water and electrical hookups are available) or cold water high pressure sprayer with low pressure detergent injection (may be portable for use in the field). Car washing brushes or mops may be used in lieu of low pressure hand sprayer for chemical decontaminant applications.

(8) Habitat measurement equipment (Global Positioning System [GPS], Digital Depth Sounder).

(9) Field Collection Summary sheets (Exhibit 1) and Field Datasheets (Exhibit 2).

(10) Chain-of-custody form (Exhibit 3).

(11) Sharpie® permanent marker in black.

(12) Powderless nitrile or latex gloves.

(13) Ice for coolers with samples.

(14) Drinking water for crew.

(15) Safety plan – the USFWS plan represents minimum requirements; agency-specific alternative plans are allowable as long as all hazards are addressed and minimum requirements are met.

(16) Bleach (any commercially available brand or strength), detergent for use with cold water pressure sprayer, or Virkon Aquatic: decontamination chemicals will only decontaminate clean surfaces, thus clean any solid surface well to completely remove any film or biological build up before application of decontaminating agents. If using cold water pressure sprayer to apply detergent in lieu of hot water pressure sprayer or bleach/Virkon, surfaces must be pre-cleaned before application of the detergent (3-5 min contact time) with a 10-second high pressure rinse.
   a. Bleach may be mixed in two strengths: 10% solutions require 10-minute contact time but 20% solutions require 5-10 second contact time.
   b. The oxidative feature of bleach deterioriates with exposure to organic material and over time, so bleach solutions must be made fresh daily in order to decontaminate DNA.
   c. Virkon must be applied in a 2% solution for DNA decontamination or reduction methods. Equipment must be fully submersed in a 2% Virkon solution for 30 minutes for complete decontamination. A 2% Virkon spray or swab application for a 10-minute contact will reduce DNA to negligible levels. Virkon mixed in solution is good for one week.

(17) Water sources: water used to mix bleach or Virkon solutions can be any source of potable water, it does not have to be DI water. Tap water may be used to wipe down surfaces or to rinse equipment such as filter funnels after a bleach soak. DI water is only required when the water used will come into contact with a filter that will be processed in the lab.

(18) Garbage bags

2.1.4 **Lotic system Site Selection Procedure (in the field)**

Specific eDNA monitoring plans that explicitly explain where and how many eDNA samples will be collected for particular water bodies will be created each year by the sampling office or agency in coordination with state partners and other agencies. These plans will contain specific sampling locations,

numbers of samples, temporal and spatial sampling details. These plans are outside the scope of this document, however, there are some critical considerations that should be followed.

(1) In lotic systems, sampling should occur in a downstream to upstream direction to minimize the potential for surface water disturbance caused by the vessels wake within the sample reach. The only exception where sampling may occur in an upstream to downstream direction would be if the nearest boat launch is upstream of the reach to be sampled or if it is not feasible to sample a system in an upstream direction (for example by kayak). Sample direction should be noted on the Field Collection Summary datasheet (Exhibit 1).

(2) Samples should be collected in two ways – transect and targeted sampling.

(a) Transect: Location of transects will be determined by the USFWS prior to the start of a sampling event. The first transect will be set across the downstream end of the reach to be sampled with subsequent transects set 500 m apart heading upstream (see exception to protocol above (1)). Transects will run perpendicular to flow, and three samples will be collected along each transect using the following scheme: one collected near the left descending bank (LDB), one in mid-channel (MC), and one near the right descending bank (RDB). Where samples are collected should be recorded in the "Habitat" column of the Sampling datasheet (Exhibit 2). Samples should be collected on the upstream side of the boat or off the bow.

- When collecting samples near the bank, be observant of wake-disturbed surfaces. To compensate for the wake created by a passing boat, samples may need to be collected 2–3 ft off the bank to obtain displaced surface film.

(b) Targeted sampling is collecting samples in the most probable places of eDNA accumulation, such as (but not limited to):

- Backwater areas
- Island side channels
- Confluences of tributary waters
- Effluent areas
- Eddies or pooled areas
- Near structures that create slack-water (e.g., sunken barges)
- Bays
- Below Lock and Dam structures
- Wind driven scumlines
- Other areas where organic material has accumulated on the water surface

Avoid sampling under or downstream of bird rookeries or storm sewer outflows since these have been shown to be vectors of eDNA from sources other than a live fish.

2.1.5 **Lentic system Site Selection Procedure (in the field)**

(1) Lake and bay samples will be selected using a blocked random sampling design including targeted collections within each block. The sampling strategy will incorporate potential focus areas based on introduction vector probability, attraction (food and habitat) and proximity to the CAWS.

(2) Once focus areas have been identified and boundaries defined, each hotspot will be divided into 8 equal sized blocks. Block size will be dependent on the hotspot boundaries, concentrating around

highest productivity area. These will then be numbered and 4 of the 8 randomly chosen for sampling.

(3) When using the filtering method, 25 water samples (2L) will be collected (100 total) for each block. Using the centrifuge method 125 water samples (50 ml centrifuge tubes) will be collected for each block. Water collection will follow a probabilistic sampling design targeting areas throughout the entire block where eDNA will accumulate.

(4) Targeted areas include backwaters, island side channels, pooled areas, below and around structures, confluences of tributaries as well as any areas where floating debris accumulates. Juvenile and age-0 Asian carp prefer shallow, productive areas like those adjacent to and within wetlands. Areas to be considered for sample collection include those with locally high productivity, such as sewage system effluents.

2.1.6 **eDNA Trailer Preparation and Field Considerations**

Routine trailer maintenance is covered in Appendix F. Prior to departure the trailer should be cleaned, decontaminated and then stocked with all supplies needed for field sampling and lab processing.

(1) Decontaminate surfaces and floors with a 10% bleach solution, allow it to soak for 10 minutes, then rinse to prevent build-up of bleach salts.
(2) Ensure all supplies needed for the field and sample processing are stocked and secure for travel.
(3) UV lights can be used for 30 minutes at the beginning of each day.

Upon arrival at the sample processing site, select an area to park the trailer that has limited risk for contamination from other activities on-going in the area. For example, avoid parking next to boat washing stations where commercial carp fishing boats will be sprayed clean.

Quality control samples should be collected from the exterior of the trailer, to be used in the event of a contaminated field control. These samples would only be processed if there is a contaminated field control.

- At the beginning and end of each sample processing day, sterile swabs or sterile filters should be used to swab the exterior of the trailer in a random location on each of the four sides, and on the door handle.
- A clean pair of gloves should be used for each of the 5 samples, and filters should be lableled with the date and trailer side ID, and then stored frozen with the samples collected that day.

2.2 Equipment Preparation

2.2.1 Purpose

In order to perform laboratory molecular analyses to detect eDNA, vessels and equipment must be decontaminated in accordance with the following protocols to eliminate introduction of outside DNA sources in the sampling regime. Bleach will only decontaminate clean surfaces, so be sure to clean any solid surface well to completely remove any film or biological build up so the bleach can destroy any DNA.

Cautions: Precautions should be made to avoid direct skin contact with bleach; bleach solution may also stain clothing or other materials. Be aware of pollutants in the aquatic environment and related health hazards.

Water used for bleach solutions may be any source of clean, potable water. Water used to rinse equipment, such as the filtering funnels or sample bottle interior, that comes into contact with the actual eDNA sample should be DI water to avoid introducing any inhibitors to the samples.

2.2.2 Equipment Procedure for Coolers and Sample Bottles

(1) A 10% bleach solution with water will be prepared in a 3-gallon low-pressure sprayer that is dedicated to the project. The bleach solution must be prepared immediately prior to use, and each time decontamination activities will be occurring.

(2) Remove mud and other biological residues from surfaces by rinsing and scrubbing. Equipment surfaces must be free of debris and other material before decontaminating.

(3) Sample transport coolers will be decontaminated with freshly made 10% bleach solution in the low pressure sprayer. Use the low-pressure sprayer to thoroughly cover the inside and outside surfaces. Allow 10 min of contact time before rinsing with water. Coolers may be left to air dry, or dried using clean paper towels while wearing clean gloves.

(4) If filtering: Sterilized disposable 2L bottles may be used for sample collection or 2L autoclavable reusable bottles sterilized in the following way may be used:

(a) NEW 2L bottles may be used straight from the manufacturer. For subsequent sampling events, send bottles to the WGL for cleaning, and WGL will return clean bottles (refer to Appendix D). For sampling agencies not working with the WGL, follow procedures b-c to properly decontaminate bottles between uses

(b) If there is no autoclave available, use a 10% bleach bath to decontaminate sample bottles.

1. In an area free of carp-contamination prepare a 10% bleach solution and soak bottles and caps for a minimum of 10 minutes. It is important to ensure that there are no air bubbles inside bottles or caps, which may require two 10-minute soaks, one with the bottle upright on each end of the closed bottle.
2. Rinse each bottle by partially filling with water and shaking vigorously so that all areas of the bottle are rinsed, repeat a minimum of three times per bottle, but be sure to rinse until there are no traces of bleach.
3. Air dry, cap, and place in sanitized coolers. NOTE: It is essential to rinse well since residual bleach would destroy any DNA collected and is a PCR inhibitor.

(c) If you have access to an autoclave, bottles need to be rinsed well with clean tap water and autoclaved.

1. Rinse bottles by partially filling with clean water, shaking vigorously with the cap loosely held over the opening. Empty and repeat a minimum of two times.
2. Place the threaded caps on the bottles and lightly screw on cap (cap will not be able to come off, but will still be able to move).
3. Place into autoclave and set cycle for 1 hour at 121°C (15 psig or 1 bar).
4. Once the autoclave cycle is complete, carefully remove bottles and allow them to cool to room temperature before fully tightening caps. If caps are tightened before allowing the bottles to cool, the bottles will warp and collapse.

If centrifuging: purchase sterile, chemical-free disposable 50-ml polypropylene tubes with maximum RCF of at least 6,000 x g. Even sterile tubes can still have traces of the chemical used to

free the plastic tubes from the metal forms during production. Some manufacturers sell sterile tubes that do not have this residual chemical in them, and these should be used to avoid introducing PCR-inhibitors from the sample tube.

(5) Once all 2L bottles have been sanitized, sample labels can be printed by the sampling agency. Sample containers will be labeled with an unique barcode ID or agency generated code (Section 1.7 for case and sample numbering) that does not indicate location (to allow blind processing). Labels will be printed on Rite-N-Rain® or some type of waterproof labels and affixed to the outside of the sample bottles/tubes.

(6) Once bottles/tubes have been labeled, they will be placed in the sterilized sample coolers in numerical order. Sample containers will be stored in the sterilized coolers until use, and will be transported only in the coolers. Although the sample ID number of the samples is not relevant except for identification purposes, collecting in consecutive order will aid in determining where samples were taken in case of a recording error.

(7) A minimum of 10% of the total number of samples collected should be cooler blanks. For offices working with the WGL, all cooler blanks must be filled by WGL. For other agencies, blanks may be filled with any city-provided tap water (If water source is a well or you are unsure of the source, use distilled or deionized water). If filtering, a minimum of one blank per cooler is required, but more may be included. If centrifuging, collect blanks to meet 10% of total number of samples collected. Note, if the sampling design for your particular body of water requires a specific sample size in order to meet a precise detection probability, then containers for control samples to meet the 10% minimum will have to be added to the total number of sample containers dictated by your sampling plan.

2.2.3 Boat and Field Equipment

This section applies to all motorized or hand-powered boats, paddles, and any associated field equipment used to collect eDNA.

(1) A set of field equipment used by staff collecting eDNA such as personal flotation devices, rain gear, hats, sunglasses, etc., should be dedicated for eDNA field work only, to avoid contamination risks. This is especially important if the same staff are involved in field work in rivers with well-established populations of Asian Carp, or if they conduct field work where they come into direct contact with Asian Carp. This dedicated gear should be stored and transported in designated containers (such as totes) so that contamination from dirty trucks or boats is avoided. After the trip, gear and transport containers should be decontaminated according to section 2.2.4.

(2) It is preferable to have designated vessels, trailers, and trucks set aside for eDNA work if possible. Even if there is designated equipment, because eDNA can be moved among sites on vessels, boats and equipment must be decontaminated prior to sampling, and between sampling sites. If complete decontamination cannot be performed between cases while in the field, choose the most preferred method of DNA reduction available in between cases (Appendix E). Upon return to the field station office, a complete DNA contamination for all associated equipment must be performed before returning to the field.

2.2.4 Boat and Equipment Decontamination Procedure

Follow steps 1-12 for decontamination or reduction of DNA on equipment surfaces before and between collections of eDNA samples from any site. Refer to Appendix E for a list of recommended

decontaminants. Use personal protective equipment (PPE) and read MSDS before use with any product. Follow equipment safety instructions and read equipment manual before using an industrial hot water pressure washer:

(1) Put on appropriate PPE. Decontamination PPE should be designated, stored separately and decontaminated after each use to prevent reintroduction of DNA to equipment, and transfer of splashed DNA around your facility.
(2) Remove equipment from boats, trucks, etc. and lay them out separately so that all surfaces of equipment will be exposed to treatment. Treat one side and then flip if necessary.
(3) Remove any environmental debris such as plant material, mud, or fish slime with brushes or gloves. If possible, perform this step at the sampling site to leave as much DNA material behind as possible. Buckets and brushes or a water pump can help rinse boat surfaces of blood and slime before leaving the water.
(4) Rinse surfaces with the highest pressure water available for the location. Surfaces must be clean for decontaminant/DNA reducers to work.

Choose a decontamination method that is appropriate for the equipment, location and services available (Proceed to steps 5, 6, 7 or 8). If equipment has been exposed to Asian carp DNA use one high pressure sprayer method ***in conjunction*** with one chemical method (steps 5 or 6 ***and*** 7 or 8) for decontamination:

(5) Use an industrial hot water pressure washer set at 212 F to decontaminate appropriate surfaces. Minimum exposure time for decontamination is 10 seconds.
(6) Apply detergent at low pressure to saturate surfaces with an industrial cold water pressure sprayer with detergent injector. Wait 3 to 5 minutes, then rinse at high pressure for 10 seconds.
(7) Mix a 10% solution of household bleach (5-8% sodium hypochlorite before mixing) in tap water in a hand pressure sprayer (low pressure saturation) or large tub (immersion bath). Spray or swab to saturate at low pressure or immerse all appropriate surfaces. Exposure time for complete decontamination is 10 minutes. Rinse with fresh water and allow surfaces to dry. Mixed solution good for one day.
    a. As an alternative to step 6 for smaller items, prepare a 20% bleach solution in a small tub and completely immerse items for 10 seconds. Rinse and allow surfaces to dry.
(8) Immerse smaller equipment in a 2% Virkon bath for 30 minutes. Metals should be immersed no longer than 10 minutes. For larger equipment, prepare a 2% Virkon solution in a low-pressure sprayer or swab and saturate surfaces. Minimum exposure time is 10 minutes. Rinse with fresh water and allow surfaces to dry. Caution: Do not aerosolize this product. Use at the largest droplet setting to avoid respiratory exposure. Mixed solution good for one week.

In absence of availability of methods for decontamination previously mentioned in this document, rinse equipment with copious amounts of water at the highest pressure available and allow to dry. Exposure to the sun or UV radiation and heat will help reduce residual DNA. Follow complete decontamination procedures beginning at step 1 upon return to field station office or at first availability.

Upon completion of methods above, follow steps 10-12 to complete eDNA clean-up:

(9) Use DNA Away and paper towels to decontaminate pens, hats, notebook surfaces, electronic equipment surfaces, truck interior and other non-saturable equipment.
(10) Contain any equipment that was not treated in bags or totes for later decontamination.
(11) Wash hands, launder or change soiled clothing.
(12) Remove PPE and decontaminate before storing separately for next use.

2.3 Motorized Sample Collection Procedure

2.3.1 Purpose

In order to perform laboratory molecular analyses to detect eDNA, samples must first be collected from the appropriate aquatic environment in accordance with the following protocols. Each morning prior to sampling, collection staff and filtering staff prior to departing will have a morning briefing that will discuss where and how the sampling is occurring. At this time a float plan and GAR model will be filed with the boat operator and collection crew and processing lead. Once the meeting is complete the processing lead will email those documents to the lead agency's main office project leader and any other necessary personnel.

Cautions: Lifejackets must be worn at all times in transport vessels (boats). Additionally, disposable powder-free latex or nitrile gloves must be worn when collecting water samples and measuring water depth and temperature. Be aware of pollutants in the aquatic environment and related health hazards. Field crews should have separate and designated field gear and outerwear that is for eDNA sampling only. This gear should be stored separately from other field gear that may come in contact with Asian carp biological material. Gear should be transported in a clean eDNA gear box so that it is not contaminated in vehicles, boats, or trailers. Follow Boat and Equipment Decontamination Procedures (above section 2.2.3)between sampling sites.

Gear (such as PFDs and hats) should be decontaminated between sampling events according to section 2.2.3. However, if any gear is compromised during sampling (accidentally falling in, or a spill, etc.) and staff feel that it may contribute to contamination of the samples, the gear should be decontaminated as soon as possible at the end of the work day, or replaced with new gear as soon as possible.

2.3.2 Water Collection Procedure

(1) Prior to launch, crew members will have reviewed this QAPP, will have signed the QAPP certification form (Exhibit 15), and will understand their assigned roles in the sample collection procedure. All sampler identification information and other field data will be recorded on the Field Collection Summary (Exhibit 1).

(2) The transport vessel will be launched from an appropriate area that allows access to the reaches to be sampled.

(3) Sampling will commence at the first transect located at the downstream end of the reach to be sampled and will proceed in an upstream direction. The only exception to this protocol is when the boat launch is located upstream of the sampling reach. Then sampling will commence at the first transect located at the upstream end of the reach to be sampled and will proceed in an downstream direction. The direction traveled for sampling should be recorded on the Field Collection Summary (Exhibit 1).

(4) It is important to avoid disturbing sediments, and avoid collecting samples where the sediments have been stirred up. If necessary, re-position the boat in a new area without disturbing sediments, make a note on the data sheet and record actual GPS coordinates where the sample was taken.

(5) When arriving at a sample location (within either a transect or targeted area), the lead sampler and sampling assistant will put on new gloves (powderless latex or nitrile). **REMINDER – Gloves must be changed before each new transect is taken to prevent cross contamination. The same**

gloves may be worn when collecting blank samples in tandem with a regular sample in a transect.

(6) Going in consecutive numerical order based on the bottle or tube labels, the lead sampler will remove a labeled sample bottle or tube from the sample cooler.

(7) Just prior to collecting the sample, the lead sampler will unscrew and remove the lid from the sample container. Do not under any circumstances touch the interior of a sample container, even with a clean glove. If you cannot grasp the container with one hand, use both hands to hold the container. Since the lid should not be set down, either hand the lid to the sampling assistant or hold the lid with the inside toward the bottle along side the bottle as you collect the sample, keeping the lid out of the water.

(8) The lead sampler will then reach over the upstream side or the bow of the transport vessel with the sample container and fill the bottle/tube by skimming the surface of the field water. The sample bottle must not be submerged or dipped beyond the upper 2 inches of the surface water for sample collection, since the intent of the sampling is to collect floating organic matter that is on the water surface. Avoid collecting large organic debris such as twigs, leaves, seeds, etc., because they cause problems in extraction. A small amount of duckweed is fine. To avoid contamination, the individual collecting the sample should avoid touching any other surfaces with the clean gloves (i.e. the gunnel) and should only handle the sample bottle and cap.

Note: Centrifuge samples are comprised of 5 replicate 50-mL tubes, which may be collected in two ways: 1) to minimize variation among offices, a sampling device may be use to collect all 5 tubes at once, as long as there is a clean place to store five caps while sampling is occurring. 2) to collect samples across a larger accumulation zone, tubes may be collected one at a time across a larger sample area within the accumulation area or along a transect, or spaced around a block, to increase detection probability. This option should be covered in the specific site plan, where specific guidance on the methods should be explicit for field crews.

(9) Once the sample container is filled (approximately 1 in. of space should be left within 2L sample bottles, and nearly full for tubes), the lead sampler will screw the lid back on to until it is tight. The closed container will then be returned to the sample cooler from which it was removed.

(9) While the lead sampler is collecting the water sample, the sampling assistant will take and record habitat measurements: water temperature, depth, GPS coordinates in Decimal Degrees, time of sample, location (e.g., left-bank decending (LBD), center, right-bank decending (RBD), and record the information on the datasheet next to the appropriate sample ID. Be sure to save the GPS coordiantes with the way-point feature of your device and to note the waypoint ID on the field datasheet.

(10) If the lead sampler pulls a transport (cooler)blank (water filled prior to trip) from the cooler, the sampler will unscrew the lid and remove to expose the bottle contents to the atmosphere for 5 sec, reseal the bottle, fully submerge the bottle in the field water, and return the bottle to the sample cooler from which it was removed. The lead sampler should relay to sampling assistant responsible for data recording that the sample was a blank, so that it can be recorded on the data sheet next to the appropriate ID. BLANKS ARE TAKEN IN TANDEM WITH THE NEXT ACTUAL SAMPLE AND DO NOT REPLACE A SAMPLE IN THAT LOCATION. If a blank has been pulled, the boat will remain at the same location and an actual sample will be taken.

(11) Steps 1 through 10 will be repeated at each sampling location until sampling has been completed for the targeted reach.

(12) Once a cooler is full, add ice to completely cover all containers. Replace ice as it melts, removing excess water only as needed, since ice water will provide better cooling than ice alone.

(13) Chain-of-custody (COC) forms (Exhibit 3) will be completed for every sample and every cooler. All samples, including blanks, will be logged onto COC forms. The forms will be collected and signed whenever the coolers are transferred between parties. If you must split samples into different configurations than listed on the original COC, make an entry for those samples on the original COC and create a new COC for any new transfer or shipping containers. Be sure that each container has its own COC that has records for the samples contained within.

2.4 Wading and Non-motorized Sample Collection Procedure

2.4.1 **Purpose**

Collection of water samples for eDNA analysis may be conducted in areas not accessible by motorized watercraft and alternative means must be employed. In order to provide accurate and creditable eDNA results, watercraft and equipment must be decontaminated in accordance following section 2.2.3 protocols to eliminate introduction of outside DNA.

Cautions: Precautions should be made to avoid direct skin contact with bleach solution which may also stain clothing or other materials. Be aware of pollutants in the aquatic environment and related health hazards. When canoeing or kayaking two people must be together either in separate kayaks or in one canoe with PFD's worn at all times and a float plan and GAR model on file with sample lead, processing lead, sample quality lead and processing quality lead and sampling agency's main office. If wading PFD's must be worn.

2.4.2 **Wading Water Collection Procedure**

(1) Collection of water samples while wading should commence at the most downstream accessible sampling site and proceed upstream. Carefully approach each sampling site slowly to avoid disrupting sediments and accumulation areas. When taking the sample the collector's position should always be down current of the targeted area.

(2) When there is no access to the downstream most site except from an upstream location field crew may enter at that point and proceed downstream carefully. Sampling will commence at the downstream most site of the reach to be sampled and proceed upstream. The entire sampling route, including direction, should be recorded on the Field Collection Summary (Exhibit 1). When traveling downstream stay out of the water as much as possible and avoid disturbing any areas of accumulation or sampling sites.

(3) The sample collector will put on clean gloves, remove the first bottle from the cooler and wade to an area of accumulation to collect the first sample. After the sample is taken, the collector will return to shore and replace the sealed bottle to the cooler. Do not under any circumstances touch the interior of a sample container, even with a clean glove. If you cannot grasp the container with one hand, use both hands to hold the container. Since the lid should not be set down, either hand the lid to the sampling assistant or hold the lid with the inside toward the bottle along side the bottle as you collect the sample, keeping the lid out of the water.

(4) When approaching an accumulation area moving downstream make a "J" like approach to disturb the water and sediment as little as possible. If approaching an accumulation area moving up stream make an "L" like approach to not disturb sediment along the bank.

(5) Change gloves and remove the next sample number going in consecutive order and proceed to the next accumulation area repeating steps 1 and 2.

(6) If sampling in a remote location a backpack container may be used. The backpack should be either a hard plastic pack/trapper's basket or a frame pack with a hard plastic storage container attached. Any backpack containers used must be able to withstand the bleach decontamination protocols and not absorb/retain water. Clean sampling bottles/tubes should be placed in a large plastic bag (i.e., heavy duty garbage bag) and closed prior to loading into the backpack. Another plastic bag placed in the backpack will be for full water samples. The person wearing the back pack will serve as the data recorder if a third person is not available. The collector will put on new gloves prior to opening and removing a sample bottle from the clean bag. Ensure the clean bag is sealed after removing a bottle. After collection, place the bottle in the bag designated for water samples, seal the bag, and remove contaminated gloves. The box of clean gloves should be sealed in a plastic bag/container between uses. Place used gloves in a separate waste bag. Place water samples on ice as soon as possible and within two hours or less of collection.

Note: Centrifuge samples are comprised of 5 replicate 50-mL tubes, which may be collected in two ways: 1) to minimize variation among offices, a sampling device may be use to collect all 5 tubes at once, as long as there is a clean place to store five caps while sampling is occurring. 2) to collect samples across a larger accumulation zone, tubes may be collected one at a time across a larger sample area within the accumulation area or along a transect, or spaced around a block, to increase detection probability. This option should be covered in the specific site plan, where specific guidance on the methods should be explicit for field crews.

2.4.3 Non-motorized Water **Collection Procedure**

(1) Kayaking/Canoeing will commence at the most upstream site (collecting samples in an upstream to downstream fashion). As you are moving downriver, try to maintain a reasonable distance from the shoreline to reduce wave action to the shoreline and accumulation areas.

(2) Clean sampling bottles (up to seven) can be placed either loosely within the boat or in a plastic trash bag depending on type and design of craft. Sample numbers of bottles should be in consecutive order and collected in order.

(3) When approaching an accumulation area moving downstream kayak #1 will make a "J" like approach not to disturb the water and sediment as little as possible. After the sample is collected and kayak #1 has moved from the collection spot kayak #2 will move into the spot to record depth, water temperature and GPS coordinates. Do not under any circumstances touch the interior of a sample container, even with a clean glove. If you cannot grasp the container with one hand, use both hands to hold the container. Since the lid should not be set down, either hand the lid to the sampling assistant or hold the lid with the inside toward the bottle along side the bottle as you collect the sample, keeping the lid out of the water.

(4) After 5-7 bottles have been collected kayak #1 will switch roles with Kayak #2 (i.e., kayak #2 is the collector and kayak #1 is the data recorder). The eDNA trailer or a filter staff member will meet the kayakers at a location downstream that is accessible to place collected water samples into a cooler with ice.

(5) At this time the kayakers will take the last of the bottles in the cooler and continue collecting within the collection reach repeating steps four and five as needed until all locations are sampled.

(6) If sampling location is in slow or no current areas (e.g., impoundments, shallow channels, etc.) sampling collection protocols should follow the lentic procedures in section XX. Craft may proceed upstream and also should collect from downwind to upwind if possible and approach all accumulation sites with minimal to no disturbance.

SECTION 3

3. SAMPLE PROCESSING (FILTERING or CENTRIFUGING)

3.1 Purpose

In order to isolate eDNA from water samples collected in the field, particulate matter must be concentrated. It can be filtered from the sample using a vacuum filtration system, or concentrated by centrifugation.

Passing each water sample through the appropriate sterile filter (1.5 micron, 5.5 cm diameter glass fiber filter) will collect particulate matter from the water sample, including sloughed cellular materials containing eDNA, on the filter paper. DNA will later be extracted from the filtered particulates and utilized in subsequent analyses.

If samples are centrifuged, matter collected at the bottom of multiple centrifuge tubes can be collected on sterile cotton swabs and eDNA can be extracted from the swab.

Cautions: Wear powder-free latex or nitrile gloves when handling samples (a glove change is required for each sample). Be careful to avoid unintentional punctures of gloves when using forceps. Punctured gloves must be changed immediately. Be careful not to touch commonly used items in the laboratory when wearing sample gloves (i.e., writing utensils, stationary lab equipment). If in doubt, change your gloves!

3.2 Filtering Procedure

Water samples collected in the field need to be filtered within 12–16 hours after the last field sample is collected.

Equipment needed:

- Manifolds (Millipore 3-place stainless steel Hyrrosol Manifold, item #XX2504735)
- Magnetic 500-ml filter funnels (Pall item #4238; VWR item #28150-496)
- Glass fiber filters, 1.5 micron, 5.5 cm diameter glass fiber filter paper (Type 934-AH; Fisher item #1827-055)
- Forceps (microforceps) – at least two pair, labeled with different colors or other identifier
- Carboy (3.5 gal or larger), for wastewater generated during filtering
- Rubber vacuum tubing (14-176-24, 1.25 in. inner diameter), double hole stopper (Fisher item #14-140P) that fits carboy opening, and glass connectors or plastic connectors (Fisher item #509557177) for connecting manifold to carboy and manifold to vacuum line
- Vaccum trap flask to protect vacuum pumps (plastic is fine), single hole stopper to fit flask and tubing to fit flask vent
- Sterile conical tubes (50mL) with caps and labels
- Sterile conical tubes (15mL) with caps and labels
- Paper towels
- Black permanent markers (e.g., Sharpie®)
- Powderless latex or nitrile gloves
- Vacuum system capable of −75 kPa vacuum
- Bleach

- 10% Bleach bath to sanitize filter funnels (use any clean, potable water source to mix bath)
- Dedicated lab equipment cleaning sink
- Waste water disposal location such as nonspecified-use sink
- Sterile bench paper
- Dedicated water bottles, one for bleach solution, one for DI water
- DI water for equipment blanks only
- Washbin for manifolds, such as a dedicated 10 qt plastic tub
- Plastic sealable bags (e.g. Ziplock®)

3.2.1 Laboratory Preparation

(1) Wash hands thoroughly prior to starting. Prepare a dedicated plastic wash bottle with 10% bleach solution for wiping down lab tables and manifold surfaces prior to processing samples. Sanitize all equipment, in freshly made 10% bleach solution, prior to starting. Collect all supplies.

(2) Rinse down each workstation with bleach solution prior to beginning the filtration process. Cover each workstation surface with sterile bench paper or clean paper towels. Bench paper must be changed between samples.

(3) Put on new powder-free latex or nitrile gloves. Prior to filtering a sample, each work station should have one black waterproof permanent marker for labeling sample tubes (or pre-printed labels), one sterile 50-mL and one sterile 15-mL plastic conical tube, sterile filter paper, one set of sterile forceps for placing filter paper on filter apparatus, one set of forceps for handling used filter paper, dedicated wash bottle with freshly made 10% bleach solution.

(4) At each workstation, connect a 3-place stainless steel filter funnel manifold to a large carboy bottle using rubber vacuum tubing, two-hole stopper, and plastic tubing connector. Connect carboy bottle to a trap flask, and the trap flask to the vacuum line with second piece of rubber tubing. Glass connectors may be used instead if available. Note, a trap flask should be between the carboy and vaccum to protect the pump from damage should the carboy get too full.

(5) Sample containers should be dried to remove all external water so that nothing will drip when the sample is being filtered or they can also thoroughly rinsed and dried completely before being brought into the trailer. Ensure there is no water trapped in the grooves between the caps and bottles by tipping bottles and drying away any water dripping out.

3.2.2 Sample Preparation

(1) Put on new powder-free latex or nitrile gloves prior to handling each sample.

(2) Remove first sample from cooler of cleaned sample bottles and make sure it is dry.

(3) Label one sterile 15-mL conical tube and one sterile 50-mL conical tube with sample number; indicate that the filter to be stored in the 15-mL tube is the equipment control by labeling this tube with a “C”. Ensure gloves that come into contact with labeling marker are not used again for handling other samples. Alternatively, labels may be preprinted and used for each tube.

3.2.3 Filtering the Equipment Control

(1) Put on new powder-free latex or nitrile exam gloves prior to processing each new sample.

(2) Place bottom portion of sterile (section 3.2.6) magnetic filter funnel equipped with rubber stopper on manifold and open vacuum line.

(3) Take designated forceps for handling clean filter paper, remove one filter and place on bottom portion of sterile magnetic filter funnel. Once the filter paper is positioned on the magnetic filter funnel, attach the upper portion of the magnetic filter funnel (i.e., the funnel) to the bottom portion.

(4) A sterile and clean magnetic filter funnel must be used for each sample. The cleaning process is described under the Equipment and Work Area Cleaning section 3.2.5.

(5) Once the magnetic filter funnel top is secured to the bottom portion, pour 500 mL of DI water the magnetic filter funnel top as a control. In order to capture any potential contaminant DNA in the funnel, be sure to pour the DI water quickly so as to immerse all the internal surfaces of the magnetic filter funnel top with DI water. Once the DI water has been poured into the filter funnel top, turn the vacuum on to draw the water down quickly and filter the material as quickly as possible. Repeat with 500 mL.

(6) Once the DI water has been filtered through the funnel, remove the filter funnel top, place it on clean bench paper dedicated for that sample. Take the forceps designated for that sample's used filter paper and grasp the edge of the filter paper. Roll or fold the filter paper until it is of a size to fit into the 15mL conical tube labeled as a control for the appropriate sample ID.

(7) Place the control filter paper into the 15mL tube, screw on top, and place tube with control sample filter paper into cold storage. If work is at a laboratory, use a −20°C non-frost-free freezer, if work is in a mobile trailer, place tubes into a cooler filled with ice during the work day. If using a cooler, be sure to use a clean sealable bag designated for control samples only to keep equipment control samples separated from field samples to avoid any contamination while in cold storage. Seal the plastic bag after each new sample is added. At the end of the day, transfer all of the tubes to a cooler lined with dry ice (replaced as needed to keep samples frozen). The freezer or cooler should be secured (i.e., locked) if samples are left for any period of time unattended.

3.2.4 Filtering the Sample

(1) Take designated forceps for handling clean filters, remove one filter and place on bottom portion of sterile magnetic filter funnel. Once the filter paper is positioned on the magnetic filter funnel, attach the upper portion of the magnetic filter funnel (i.e., the funnel) to the bottom portion. Avoid touching the funnel base with clean forceps. If forceps accidentally touch the base, get a new pair of forceps for handling clean filter paper. These designated clean forceps and the box of filters must be kept in a location far enough away from the filtering area to prevent them from becoming contaminated during filtering. If at any time water is spilled or splashed near the filters or forceps, get a new box of filters and a new pair of forceps.

(2) Take the sample bottle and gently shake to distribute the contents within the sample evenly.

(3) Once the magnetic filter funnel top is secured to the bottom portion, turn on the vacuum and then pour approximately one-third of the sample into the magnetic filter funnel top.

(4) Once the one-third portion of the sample has been filtered through the funnel, *continue filtering until the filter is nearly dry*. Remove the filter funnel top. Take the forceps designated for used filter paper and grasp the edge of the filter paper. Neatly fold the filter paper once to fit into the 50mL conical tube labeled for the appropriate sample ID.

(5) Repeat steps 1–4 at least two more times or until the entire sample has been filtered. After all the sample has been filtered, DI water from a wash bottle may be used to rinse any particulates attached to the sides of the magnetic filter funnel onto the filter paper. NOTE: since this water is touching the DNA sample DI water is better than tap water.

(6) Place the tube with field sample filter papers into cold storage. If work is at a laboratory, use a −20°C non-frost-free freezer, if work is in a mobile trailer, place tubes into a cooler filled with ice during the work day. Use a sealable plastic bag designated for field samples only, to keep the sample tubes organized and separated from equipment control samples. Seal the plastic bag after each new sample is added. At the end of the day, transfer all of the tubes to a cooler lined with dry ice (replaced as needed to keep samples frozen). The freezer or cooler should be secured (i.e., locked) if samples are left for any period of time unattended.

(7) The number of filters used to process the sample is up to the discretion of personnel processing the sample, however in the DNA lab, filters must be extracted one at a time. Therefore, if the water sample exhibits an excessively slow filtration rate, multiple filters should be used. Also up to the discretion of the personnel processing the sample is the amount of sample water to run through a single filter. A general rule is to run one-third of a 2L sample through a single filter; however, if the sample water is extremely turbid, for example, less water should be put through a filter. At the other end of the spectrum, if the sample water is extremely clear, more than one-third of a 2L sample may be run through a single filter. Do not place more than 10 filters into a single 50-mL tube. If more than 10 filters are generated, use additional 50-mL tubes, making sure they are clearly labeled with the sample ID and make a note on the data sheet.

(8) On the field datasheet (Exhibit 2) next to the appropriate sample ID, mark the time (use AM PM or military time for clarity) of filter completion and the initials of the person that processed the sample.

(9) Change gloves and sterilize the workstation between samples. Repeat steps 1–8 until all samples have been processed.

(10) When filtering, if the water collection carboy becomes full, disconnect the carboy from the vacuum and manifold and dispense water in a sink separate from the one used to clean equipment. Once emptied, reconnect the carboy to the vacuum and manifold and proceed with the filtering process. **CAUTION: Be sure to open manifold valve and turn off the vacuum air supply when disconnecting and connecting the carboy so as to prevent explosion of the glass.**

(11) If sample is accidentally spoiled during the filtering process (e.g., bleach was used to rinse filter funnel instead of DI water, forceps from previous sample used, etc.), immediately throw away ruined samples. If portions of the sample are still viable, place in 50mL conical tube. On the outside of the sample tube, label with the amount of the viable sample (e.g., 2/3 sample). On datasheet, label with the same information (e.g., 2/3 sample) next to appropriate sample ID. Note the reason for the ruined or diminished sample.

3.2.5 Equipment and Work Area Cleaning After Filtering Each Sample

(1) Fill a 500mL glass beaker with 10% bleach solution. Forceps designated for used filter paper must be switched out for each sample. Used forceps will be placed in beaker with 10% bleach solution for

a minimum of 10 min for sterilization. Once sterilized, remove forceps from bleach solution and rinse thoroughly before use.

(2) Fill at least a 10-qt plastic tub (e.g., Rubbermaid® plastic storage bin) with 10% bleach solution. Once a sample has been processed, the filtering apparatus must be dismantled (i.e., the magnetic filter funnel should be separated into the upper and lower parts), rinsed to remove any particles and/or film, and placed in the plastic tub with the 10% bleach solution for a minimum of 10 min for sterilization. Ensure there are no air bubbles preventing the bleach solution from fully contacting the entire filter cup interior. Once sterilized, remove the two parts of the magnetic filter funnel and thoroughly rinse before use. Rinsing should continue until all residues and scent of bleach can no longer be detected.

(3) In between each sample, dispose of bench paper. Wipe down surface with 10% bleach solution using wash bottle and paper towels. CHANGE GLOVES! Cover work station with new bench paper.

If any deviations from this QAPP occurred during water sample collection in the field or during processing of samples, clearly describe the deviations on Exhibit 16. Even if there were no deviations from the QAPP, Exhibit 16 forms should be sent to the eDNA Project Coordinator following a sampling event to satisfy reporting requirements in section 1.6. Be sure to note any changes in personnel from the pre-trip plan on Exhibit 16 when the report is filed.

3.3 Centrifuging Procedure

Water samples collected in the field need to be centrifuged within 12–16 hours after the last field sample is collected.

Equipment needed:

- Sterile, chemical-free centrifuge tubes made of polypropylene that can withstand 6000 xg with caps and labels.
- Paper towels
- 95% ethanol or 70% isopropyl alcohol (mix prior to trip with purchased molecular grade water)
- A reuseable bottle-top re-pipettor for dispensing alcohol
- Black permanent markers (e.g., Sharpie®)
- Powderless latex or nitrile gloves
- Refrigerated centrifuge(s) with rotors and adaptors for 50-ml tubes (Fisher item # listed for: Sorvall Legend XTR centrifuge; rotor GS25F7087G item #75-033-607; 750 ml round bucket GS25F7087G item #75-003-608; 50-ml tube adaptors item #75-003-638)
- Bleach
- 10% bleach bath for decontaminating plastic centrifuge tube adaptors
- 20% bleach bath for decontaminating centrifuge tubes before processing
- Rinse bath of water to rinse containers before processing
- Dedicated lab equipment cleaning sink
- Waste water disposal location such as nonspecified-use sink
- Clean bench paper or clean paper towels
- Dedicated water bottles: one for DI water; one for bleach solution
- Washbin for centrifuge adaptors and buckets, such as a dedicated 10 qt plastic tub

3.3.1 Laboratory Preparation

(1) Hands must be washed thoroughly prior to starting. A dedicated plastic wash bottle with 10% bleach solution should be prepared for wiping down lab tables and other surfaces prior to processing samples. All equipment must be sterilized and all supplies collected prior to starting.

(2) Each workstation must be rinsed with bleach solution and the surface covered with one or more clean paper towels or bench paper prior to beginning the centrifuging process. Paper must be switched between each batch of sampling sites. New powder-free latex or nitrile gloves must be worn for processing each batch. Prior to centrifuging samples, each work station should have pre-printed labels or one black, waterproof permanent marker for labeling sample tubes, a wastewater container (with lid), and a dedicated wash bottle with 10% bleach solution, and a dedicated alcohol bottle with 95% ethanol or 70% isopropanol. At least a 10-qt plastic tub (e.g., Rubbermaid® plastic storage bin) should be filled with 20% bleach solution for the sterilization bath inside the lab.

(3) At each workstation a refrigerated centrifuge set at 4^0 C. If in mobile trailer, check horizontal alignment, trailer should be as level as possible.

(4) A decontamination station with a 20% bleach bath to decontaminate racks and tubes prior to processing.

(5) Centrifuge tube racks should be available for organizing and working with sample tubes. One set of racks can be used with samples prior to decontamination, and a second set of racks should be decontaminated by dipping in the 20% bleach bath and rinsing with water. Take care to place only decontaminated tubes in the clean racks. Be sure tubes are dried well after their sanitizing dip to avoid any contamination of the samples during processing.

3.3.2 Sample Preparation

New powder-free latex or nitrile gloves must be worn prior to handling each sample set.

Process tubes in sets of replicates, until a full centrifuge batch is prepared.

A centrifuge equipment control comprised of 50 ml of tap water should be included with each batch of tubes in each centrifuge. It should be labled with the range of sample numbers included in the batch. For example, centrifuge equipment control sample number 10001-10005 was included in the centrifuge with field samples 1 through 5 from case number 10000. This equipment control is in addition to the field control, and should be made by the sampling office or agency.

3.3.3 Centrifuging the Samples

(1) 50-ml tubes should be removed from the transport cooler and examined to ensure the meniscus of the water is close to the 50 ml line and caps checked and tightened. Sample containers should be submerged to the bottom of the cap in the 20% bleach for 10 seconds and then completely dried before being moved into the processing area.

(2) Place the 50ml tubes that have been cleaned and dried in the refrigerated centrifuge set at 4 ^{0}C. Tubes must be evenly distributed within the centrifuge to maintain the rotor balance. NOTE: Always follow the manufacturer's guidelines for centrifuge operation. Remember to include one equipment control tube with each full centrifuge. It is also important to keep replicate tubes for each sample together in batches. Do not split a sample between batches, otherwise it is too difficult to trace equipment control results through the lab.

(3) Once 50 ml tubes are in position, close and secure centrifuge lid. Set centrifuge to spin the samples for 30 min at max speed (~4500-5000 xg) and begin centrifuging the samples. During this period the other tubes may be decontaminated and placed on a sterilized surface or rack. DO NOT PLACE DECONTAMINATED TUBES ON ANY SURFACE OR RACK THAT HAS NOT BEEN STERILIZED.
(4) Once samples have been centrifuged the eDNA will be on the bottom of the tube. Wearing a new pair of gloves for each set of tubes comprising one sample, carefully remove cap and GENTLY pour off water into a wastewater container. A carboy is useful to prevent splashing, or a plain bucket with bleach in the bottom will also work. Change gloves after each sample set.
(5) Add ~5 ml of 95% ethanol or 70% isopropanol to the tube to stabilize the eDNA. Replace cap and swirl alcholol around tube covering the entire internal wall. Centrifuge the samples with alcohol for 10 minutes (or more as needed if pellet is too loose) at max speed (~4500-5000 xg).
(6) Decant excess alcohol until the level of the alcohol is within the conical end of the tube. Place tubes back into a sterilized styrofoam container. Change gloves between sample sets.
(7) When styrofoam racks are full, put them in plastic bags so that the tubes are secured in the rack. Tape bags closed and sign across the tape where it overlaps. Place in box to be shipped. NOTE: If a stabilizing solution (i.e., ethanol) is not used eDNA samples must be frozen (traditional commercial freezer or −20 °C freezer) or placed in cold storage. To conserve dry ice, samples may be placed in a cooler filled with ice during the working day. At the end of the day, quickly transfer all samples into a dry ice cooler for long-term storage. Be sure to monitor temperature twice a day and replenish dry ice as needed.
(8) Prior to centrifuging the next batch of 50 ml samples remove the 50 ml tube adaptor inserts and examine for any water or debris. If anything is found, place in 10% bleach bath for 10 minutes, rinse well to remove all residual bleach, dry with new paper towels, and replace. If adaptor inserts are clean and dry only perform this step during daily lab cleanup.
(9) On the field datasheet next to the appropriate sample ID, record the time of centrifuge completion and the initials of the person that processed the sample.

Steps 1–9 should be repeated until all samples have been processed.

As always, gloves must be changed and the workstation sterilized between batches.

When the wastewater collection carboy is full, it should be disposed of in a sink separate from the one used to bleach equipment. If a second sink is not available, dispose of the wastewater in any drain that is connected to a sewage treatment facility or system.

If a sample is accidentally spoiled during the centrifuging process (e.g., the pellet was lost during decanting, bleach was squirted into a tube, or cross-contamination is suspected), it should immediately be thrown away. Record on the corresponding datasheet the appropriate sample ID as well as the reason for the ruined sample.

If any deviations from this QAPP occurred during water sample collection in the field or during processing of samples, clearly describe the deviations on Exhibit 16.

Even if there were no deviations from the QAPP, Exhibit 16 forms should be sent to the eDNA Project Coordinator following a sampling event to satisfy reporting requirements in section 1.6. Be sure to note any changes in personnel from the pre-trip plan on Exhibit 16 when the report is filed.

SECTION 4

4. SAMPLE SHIPMENT

4.1 Purpose

Samples must be maintained on dry ice or in a -80 freezer until they can be shipped to the DNA processing lab (WGL lab in Onalaska, WI) as soon as possible. Temperature should be recorded twice a day during sample storage and immediately prior to sealing the shipping container (Exhibit 7). If samples were centrifuged, samples may be shipped at the end of the sampling event. The Processing Leader is responsible for ensuring that samples are properly packed and shipped according to the procedure below.

Please note: the COC forms are as important as the samples themselves. If COC forms are not filled out properly, then sample integrity is lost and the samples cannot be processed because their custody cannot be accounted for. Therefore, please be sure to accurately and completely fill out the COC forms. If you have questions, do not hesitate to call the eDNA Program Coordinator or the eDNA laboratory.

Cautions: Wear gloves and use caution when working with dry ice.

4.2 Shipping Procedure

(1) If filters: Corrugated boxes (minimum outer dimensions 12" X 12" X 12") with styrofoam cooler inserts will be prepared for shipment. The number of boxes to prepare depends upon the number of samples collected (e.g., a 120-sample collection will require more boxes for shipping than a 50-sample collection). Filtering equipment controls should be placed into separate plastic bags during shipping, but centriguge equipment controls can be mixed in with samples.

If centrifuge tubes: Any shipping containers that will hold the tubes and prevent damage to the tubes will work.

(2) For filters or non-preserved centrifuge samples: The bottom of the coolers will be lined with solid blocks of dry ice (approximately 1–2 inch thickness). Oven-mitt type gloves must be worn by personnel that are handling dry ice to protect hands. If block dry ice is not available, pellets may be used, but avoid powder or flake.

(3) For filtered samples: Remove the 15mL conical tubes with control filter paper from secure (i.e., locked) −20°C freezer and place in clean 1-gal resealable bag (e.g., Ziploc®). Multiple bags may be used if the entire sample does not fit in one bag. Seal over the opening of all bags used with tamper-evident tape (e.g., Evidence Tape – NC9709516). The individual packing the samples should sign the tape. Be sure to remove as much air as possible from all resealable bags used before sealing.

Be sure bags are secured so that they are tamper-evident. There are several ways to do this, here are two: 1) Close and seal bags, removing as much air as possible. Wrap tamper-evident tape or clear tape around the bag in two perpidicular directions so that the bag cannot slide out of the tape. If you don't use tamper evident tape, be sure to sign across where the tape meets itself with a permanent marker. 2) Close and seal bags, removing as much air as possible. Place tamper evident tape parallel to the bag seal, securing with three (3) staples. This option does not allow for clear tape with a signature.

When ambient temperature is above 60^0F, more dry ice is needed for shipping than when it is cooler. If possible use both block and pellet dry ice to ensure sample integrity when shipping during

warm weather. Place a ~10-lb block of dry ice on the bottom of cooler. Place a bag of samples on the block, layer approximately 1 in. of dry ice pellets on top of bag before placing another bag in the cooler. Repeat until only 2 inches of space is left at the top of cooler and fill the space with dry ice pellets. If pellets are not available, use a minimum of 20 pounds of block dry ice. Leave the bottom block whole, but break up the other block and distribute among the bags of samples. Before closing the styrofoam cooler, record the inside temperature on the datasheet (Exhibit 2). Place the styrofoam lid on top of the sample contents and seal with tamper-evident tape, ensuring the tape crosses the lid and body of the cooler. If tamper-evident tape is not available, use packing tape and sign across where the tape overlaps as well as across were the lid meets the body of the cooler.

For centrifuge samples: Be sure tubes are securely closed to prevent leaking. If tubes have been chilled, lids may become loose, so double-and triple-check before packing. Place tubes back into the foam rack in numerical order. Place the rack of tubes back into the plastic bag the tubes came in, wrap it tight to keep tubes from falling out, tape it closed, signing across the tape. Pack racks of tubes into boxes, using packing material to keep them from shifting. These can be shipped at ambient temperature since they are preserved in alcohol.

(4) Fill out a COC form <u>for each shipping container</u> where the most recent listing lists exactly and only the samples shipped in that particular container (equipment controls are separate samples from the field samples, so they must be accounted for on COC forms in the containers in which they are shipped). The individual employee packing and sealing the containers should list their name in the "from" line, be sure to include agency and print clearly. The container should be packed and released on the same date. Sign and place COC forms (Exhibit 3) in a clean 1-gal resealable bag, place evidence tape across the seal, and place the bag on top of the cooler before closing the corrugated shipping box and sealing with packing tape.

(5) Repeat steps 1–4 for 50mL conical tubes and additional boxes. **<u>Each box MUST</u>** have a separate signed COC form included to document the specific samples included therein. If you must split samples into different configurations than listed on the original COC, make a new entry for those samples going into one container on the original COC and create a new COC for any new shipping containers.

(6) Fill out a Federal Express (FedEx) air bill shipping label with appropriate information. Be sure to affix a dry ice warning label (can be purchased in rolls from FedEx, or printed from the internet) with the amount of dry ice in each cooler on the cardboard shipping container. On the label be sure to designate FedEx Overnight Express (delivery is usually the following day between 8 and 10 AM) as well as to identify the weight of the dry ice in the package. When ready, drop off at FedEx or call FedEx (1-800-463-3339) for pickup. Be sure to tell the operator that the package contains dry ice and ask for an approximate pickup time. Be sure to record tracking numbers for all boxes being shipped.

(7) Items will be shipped to the following address:

Whitney Genetics Lab
555 Lester Avenue
Onalaska, WI 54650
608-783-8444

See item 8 for WGL contacts

(8) Once items have been picked up for shipment, at least one person at WGL must be contacted and notified of approximate delivery date and time. At least one of the following personnel should be contacted via telephone and email regarding shipment, continue calling down the list until you speak with a human on the phone. Following shipment, email approximate delivery time, date and tracking numbers to the person you talked with and the person who will be receiving the delivery at WGL:

Emy Monroe
Phone: 608-783-8402
Email: emy_monroe@fws.gov

Maren Tuttle-Lau
Phone: 608-783-8403
Email: maren_tuttle-lau@fws.gov

Jennifer Bailey
Phone: 608-783-8451
Email: jennifer_bailey@fws.gov

Nick Grueneis
Phone: 608-783-8404
Email: Nikolas_Grueneis@fws.gov
Nick Berndt
Phone: 608-783-8419
Email: Nicholas_Berndt@fws.gov

Kyle Von Ruden
Phone: 608-783-8411
Email: Kyle_VonRuden@fws.gov

(9) Upon receipt of the samples at WGL, if the samples were shipped with dry ice, the inside temperature of all the coolers must be taken and recorded. Samples shipped with dry ice that have remained at room temperature (approximately 20°C) for more than 24 hours will be discarded, and the sample names and reason(s) for discarding will be noted in the laboratory log. Samples that are shipped at ambient temperature do not need a temperature recorded upon receipt.

(10) Personnel receiving the shipment must immediately sign the COC form, scan it and email it back to the sender of the COC. Make sure to cc the Data Reporting Specialist for the project so that proper documentation can be recorded. Receiving personnel should also call the sender and sampling leader if they do not have email access in the field.

SECTION 5

5. DNA ASSAYS

5.1 General Quality Assurance and Chain-of-Custody Considerations

(1) Any change to described DNA handling, storage, or processing procedures must not result in reduction of eDNA sensitivity relative to current values and must be cleared with the eDNA Processing Leader. The current protocol produces the following results at different concentrations of purified DNA amplicons [1] in sterile water:

Species	Purified DNA amplicon (copies/µl water)	Purified DNA amplicon (ng/µl water)
Bighead Carp	207	3.30×10^{-8}
Silver Carp	7	7.25×10^{-10}

(2) Each stage of eDNA genetic processing procedures (eDNA sample extraction, PCR setup, and post-PCR processes) should be performed in a separate room in order to minimize the risk of sample cross-contamination. If separate rooms are not available, extractions and PCR set up can be combined in one room, ONLY if PCRs are set up in a PCR hood with a hepa filter and UV light. All post-PCR processing *must* be in a separate room.

(3) Every effort should be made to ensure that equipment, work areas, and solutions are free from DNA contamination. All surfaces should be wiped clean with 10% bleach solution (or commercial DNA eliminating solution such as DNA Away) before and after use. If equipped with UV lamps, clean lab rooms or PCR hoods should be irradiated with UV light 30 minutes at the beginning and end of the work day.

(4) All new microcentrifuge tubes (non-sterile) and glassware used in the lab must be autoclaved at 121˚C for 20 min before being used. Any re-used items must be soaked in a freshly made 10% bleach solution for 10 minutes followed by a thorough rinse.

(5) Good housekeeping policy should be practiced at all times. Reagents that have passed expiration dates should not be used, nor should any reagents that have been kept at incorrect storage temperatures for a significant length of time. All reagents, reaction tubes, etc., must be clearly labeled. Records of batch numbers of all reagents used in individual assays should be made whenever reagents are signed out from the designated freezer. The temperatures of cold storage units must be monitored once a day, using the forms given in Exhibits 7-8.

(6) Positive and/or negative reactions should be used to test all new batches of critical components prior to or concurrent with their application to eDNA samples.

(7) Standard sterile techniques should be used in the DNA laboratory to prevent the unintended transfer of DNA between surfaces, and to prevent cross-contamination between samples.

[1] As reported in Jerde et al., 2012. Response to Casey et al.'s sensitivity of detecting environmental DNA comment. Conservation Letters 0:1-2.

Contamination can adversely affect the outcome of a case; therefore, it is essential that the laboratory have procedures in place to limit, recognize, and address contamination.

(8) Gloves (e.g., powder-free nitrile or latex) must be worn throughout sample processing. At a minimum, gloves should be changed at the completion of each step of the process. If gloves become contaminated or if contamination is suspected, discard them and replace them with new ones. For example, gloves should not be worn when using or handling keyboards, notebooks, pens, telephones, etc. and must be replaced immediately before recommencing bench work.

(9) Centrifuge tubes before opening the reagents. Uncap and close tubes carefully to prevent aerosol contamination.

(10) Ensure that centrifuges are always balanced when centrifuging samples.

(11) Ensure that all equipment, including paper, pens, and lab coats, are dedicated for use only in that particular laboratory (e.g., laboratory coat for each stage of procedure rooms. Workbooks that have been in contaminated areas shall not be taken into clean PCR areas. A Project Lab Book should be kept in a room separate from the DNA Extraction Room and DNA PCR Room. Each room (Extraction, PCR, Post-PCR) should have note-taking materials (e.g., loose-leaf paper, networked tablet PCs) that can be transported or viewed for consolidation in the Project Lab Book. Other solutions for preventing contamination of sensitive areas via lab notes may be used following approval by the eDNA Processing Leader. Any changes should be incorporated into a revised QAPP. Laboratory notes/notebooks should:

- Be written or printed on tamper-proof paper (e.g., does not exactly photocopy).
- Have lab book identification, with consecutive numbering, dates, and signatures (of the note-taker) on each page.
- Be made using permanent ink. Special pens may be required for certain paper types.
- Have any changes to notebooks be dated and initialed by the person who made the change. Any incorrect information should have a single line drawn through it and not be completely obscured.
- Contain all data images (e.g., gel photographs, denaturing curves, DNA sequence electropherograms). Images should be permanently affixed to the notebook and signed across both the edge of the insert and the page.
- Be kept in a locked drawer or cabinet with restricted access when not in use.

(12) A log of all batches of critical components should be kept. This log should include material safety data sheets (MSDS) and product information sheets. Dates of receipt, opening, testing, and disposal for each component should be recorded in the log.

5.2 Quality Control for Sample Custodian Procedure and Storage

(1) An internal log book should be kept for all samples. Tamper-proof paper should be used. The log book should be kept in a locked drawer or cabinet when not in use.

(2) Separate freezers should be designated for storage of (a) concentrated water samples, (b) DNA extracts, (c) PCR and sequencing product, and (d) PCR, cloning, and sequencing kit components. A dedicated refrigerator should also be maintained for any PCR, gel electrophoresis, cloning, and sequencing kit components that require 4°C storage.

(3) Maps or other designations of the location of samples within freezers should be maintained.

(4) All items in freezers should have indelible ink identifications.

(5) All freezers should have non-universal locks or marine brackets attached that can be used with keyed locks, or be housed in a secure facility.

(6) All samples placed into or removed from freezers should be signed for on freezer log or ambient storage log (see Exhibits 7, 8, and 9).

5.3 Physical Separation of Pre-PCR and Post-PCR Assay Stages

5.3.1 The eDNA Extraction Room

- Extraction of DNA must be performed where PCR products and stocks of cloned material are not handled.
- A PCR hood with a built-in ultraviolet (UV) light and HEPA filter may be used to further isolate DNA extraction kit solutions and elutes from ambient DNA.
- A complete separate set of necessary laboratory equipment, consumables, and laboratory coats should be dedicated for use in DNA extraction.

5.3.2 Pre-PCR Room

- To prevent carry-over of amplified DNA sequences, PCR reactions should be set up in a separate room from that used for post-PCR manipulations.
- A completely separate set of necessary laboratory equipment, consumables, and laboratory coats should be dedicated for the specific use of pre-PCR manipulations.
- Reagents and supplies should be taken directly from clean storage into the PCR area and should never be taken or shared with areas in which post-PCR analyses are being performed. Equipment such as pipettes should never be taken to the post-PCR area after use with amplified material.
- A sign-in log system should be implemented for use of the thermal cyclers (PCR machines), including the run name, plate orientation, and the number of thermal cycler heads.

5.3.3 PCR Product Analysis Room

This is the post-PCR Room where post-PCR manipulations are performed, including agarose gel electrophoresis of products and sequencing of presumptive positives.

- This room is a contaminated area; therefore, **no** reagents, equipment, laboratory coats, etc. from this room should be used in any of the other lab areas.
- A biological or PCR-type hood may be used for setting up cloning or sequencing reactions.

5.4 Receipt of Filters/Tubes

5.4.1 Source

Water samples have been taken in the field according to Section 2 and filtered or centrifuged according to Section 3. Samples have been shipped to WGL and personnel assigned to the WGL eDNA Team have received packages from the overnight service. NOTE: filters are shipped cold, but alcohol-preserved centrifuged samples are shipped at ambient temperature.

(1) Upon receipt of samples from the eDNA sample filtering team, the shipped box(es) should be opened and if shipment was on dry ice, the temperature inside each box recorded. The general condition of the box(es) should also be recorded.

(2) To measure temperature upon opening the box, either (a) place a glass thermometer inside the Styrofoam container, replace lid, and leave the thermometer in place for at least 2 min before removing it and immediately recording temperature or (b) immediately aim an infrared laser thermometer at the samples and press the MEASURE button to record the temperature inside the cooler.

(3) Place filtered samples in filter sample storage freezer (–80°C) and log samples on freezer sheet (Exhibit 8). Place centrifuged sample tubes in ambient sample storage (shelves) and log samples on bench-top log sheet (Exhibit 9).

(4) Sign and date the COC forms that accompanied the samples. Place them in a designated file; a copy should also be provided to sampling agency. If the forms were inside sealed bags, slit the bag to remove the COC forms. Note any condition issues (broken tape or seals, damaged containers or bottles, etc.) with the samples on the COC forms. Note any samples that must be discarded due to condition issues and the reason for discard.

(5) Enter sample data into internal sample log book (or LIMS), including noting any samples that are being discarded and that should not be analyzed, and create new WGL COC form for samples. Note any observations about samples such as condition issues. Store COC forms in a secure area.

(6) Alert Sampling and Processing Leaders that samples have been received. Use e-mail addresses with return/receipt requested and directly contact via telephone; team contacts are listed in Appendix A. This reporting must be done within 1 hour of receipt.

5.5 DNA Extraction from Filters or Tubes

5.5.1 Source

Filters or tubes from eDNA sampling have been received by WGL eDNA Team and logged. Filter samples should be in designated −80°C freezer, centrifuged sample tubes may be in ambient storage or if they are not preserved with alcohol in designated -80°C freezer.

5.5.2 DNA Extraction Quality Assurance and Chain-of-custody

At this stage, a critical component of quality control should be to correctly label all sample extraction processing tubes so that there is no question about the origin of samples.

(1) Bench areas in DNA extraction laboratory and PCR-type hood (if used) should be wiped before and after use with 10% bleach. Validated, commercially available sterilization reagents, such as LookOut® DNA Erase®, may be preferred. Extraction rooms should be irradiated with UV lights for 30 minutes prior to use.

(2) After an item or surface is cleaned with bleach, it must be rinsed with purified water or alcohol to prevent the build-up of sodium hypochlorite crystals. Instruments or equipment cleaned with bleach must be rinsed to avoid corrosion.

(3) It is common practice for moisture barrier paper towels to be placed on the bench top while processing samples to act as a barrier. The paper barriers must be changed and the bench top cleaned between sample batches.

(4) Centrifuges, thermal cycler, tube racks, pipettes, and any other equipment used for the extraction process should be cleaned before and after each use.

(5) Instruments such as forceps and scissors should be cleaned just prior to use. Sterile disposable equipment should be opened just prior to sample processing and discarded after one use.

(6) When using pipettors, use filtered tips and never allow the liquid in a pipette tip to rise up to the barrier.
- Do not rest the pipette on a dirty surface.
- Avoid cross-contamination by changing pipette tips after each use.
- Watch that the tip -- and only the tip -- is allowed to go into a bottle of reagent, never the pipette itself.

(7) Record all solution batch/lot numbers used for reactions in lab notes.

(8) Always mix tubes by vortex or finger flicking, and then briefly spin down tubes before opening.

(9) No deviations to the DNA extraction protocol are allowed without written approval by the eDNA Processing Leader. Any errors in processing should be noted in the laboratory log. Samples affected by errors in the extraction protocol should be clearly identified.

5.5.3 Alcohol evaporation from centrifuged samples procedure

(1) Centrifuged samples preserved with alcohol must have the alcohol evaporated away before extracting the samples. Prepare laminar flow hood by wiping down the work surface with 10% bleach or DNA away and/or use the UV lamp for 15 mintues.

(2) Remove samples from freezer or ambient storage; note on freezer/storage and sample log (see Exhibits 7-9).

(3) Move samples in tube racks to the laminar flow hood. Carefully remove tube lids and place in the same order as the samples next to each rack. Turn on the air flow and leave the samples to dry until all traces of ethanol or isopropanol smell are gone, because these are both PCR inhibitors.

(4) Include a hood negative control sample for each extraction batch set out to dry. For our extraction batches of 30, this equals 27 field samples, an extraction positive and negative, and one hood negative control. Make a negative hood control by placing an empty, sterile centrifuge tube in the racks with the lid removed alongside the field samples.

(4) Positive and negative extraction controls should be added to each eDNA extraction procedure batch.

- Before proceeding with extraction, a positive control swab is prepared by pipetting 300 µl of Silver and Bighead carp cell lines or tissue slurry directly onto a sterile swabs in 1.5-ml flip-top tubes. Alternative species (e.g. – sturgeon) may be used as the positive control to reduce risk of sample contamination from carp tissue. Alternative species must have PCR primers that (1) do not cross-react with carp DNA and (2) can be run on the same thermocycler settings as carp samples. A batch of extraction positives can be prepared ahead of time and frozen at -20°C.

- Additionally, an extraction negative control sample should be prepared by pipetting 300 ul of sterile lab DI water onto a sterile swab in a 1.5-ml flip-top tube. A batch of extraction blanks can be prepared in advance and kept frozen at −20°C

- For every extraction batch of filter samples or centrifuged samples processed, conduct DNA extraction (below) on one frozen, sterile extraction negative control and one prepared positive control.

(5) For all samples and cooler, equipment, and extraction controls, follow the DNA extraction protocol detailed below.

Cautions: As with all components of eDNA processing, quality control and sterilization procedures must be carefully followed in order to avoid contamination of downstream procedures.

5.5.4 PowerWater Procedure for *filters*

(1) Remove samples from freezer if filters, note on freezer and sample logs (see Exhibits 7-9).

(2) Be sure you have added positive and negative extraction controls to each eDNA extraction procedure batch.

(1) Before proceeding with extraction, a positive control filter is prepared by pipetting 300 µl of a mixed slurry of homogenized Silver and Bighead carp tissue or cells directly onto a sterile filter paper. Alternative species (e.g. – sturgeon) may be used as the positive control to reduce risk of sample contamination from carp tissue. Alternative species must have PCR primers that (1) do not cross-react with carp DNA and (2) can be run on the same thermocycler settings as carp samples .
(2) Additionally, an extraction negative control sample should be prepared by placing a new filter paper in a new sterile 1.5mL microcentrifuge tube (MCT) and pipetting 300 µl of sterile lab DI water directly onto the filter. A batch of extraction blanks can be prepared in advance and kept frozen at −20°C.
(3) For everyextraction batch of filter samples processed, conduct DNA extraction (below) on one frozen, sterile extraction negative control filter and one prepared positive control filter.

(3) For all samples and cooler, equipment, and extraction controls, follow the DNA extraction protocol detailed below. For each filter, you will need one spin column and 4 1.5/1.7 ml lab supplied MCT and 5 Powerwater collection tubes.

This DNA extraction utilizes the PowerWater DNA Isolation Kit (MoBio Laboratories, MoBio Inc.) and the protocol is adapted from the manufacturer's protocol (http://www.mobio.com/images/custom/file/14900.pdf).

(a) Place Solution PW1 in a 55°C water bath for 5–10 min to dissolve any precipitates that have formed at room temperature. Remove Solution PW1 from the water bath immediately prior to use.

(b) Remove the appropriate filter sample from −20°C freezer and transfer the filter(s) to a labeled 5mL PowerWater Bead Tube. If DNA will be extracted from multiple filter samples, continue to remove each filter sample from −20°C freezer immediately prior to filter transfer. Each PowerWater Bead Tube will hold up to two filters per sample. If any filtered water samples required more than two pieces of filter paper, split the filters into two per PowerWater Bead Tube.

Note: Change gloves between the transfers of each sample to avoid cross-contamination of samples.

(c) Add 1mL of Solution PW1 to the PowerWater Bead Tube and secure the cap tightly. Mount the tube on a vortex adaptor (MoBio Inc.) and vortex on high for 5–10 min, or until the contents of the bead tube appear liquefied. Times can vary depending on the number of filter papers being extracted. A bead beater can be used on a large batch of bead tubes if available.

(d) Centrifuge the tubes at 4,000 x *g* for 8 min at room temperature. Ensure centrifuge is balanced before centrifuging. Transfer 650-800 µl supernatant using a 1mL pipette to a labeled 1.7mL lab supplied MCT.

(e) Centrifuge tubes at 13,000 x *g* for 1 min and carefully transfer 650 µl of the supernatant with a pipette into a new labeled 1.7 mL lab supplied MCT. Be sure to avoid any beads or filter debris.

(f) Add 200µL of Solution PW2, vortex briefly, and incubate at 4°C for 5 min. It should appear cloudy.

(g) Centrifuge the tubes at 13,000 x *g* for 1 min and carefully transfer 650 µl of the supernatant with a pipette into a new labeled lab-supplied 1.7 mL MCT.

(h) Add 650µL of Solution PW3 and vortex briefly. Load 650µL of supernatant onto a spin filter, place spin filter into a MoBio 2mL tube and centrifuge at 13,000 x *g* for 1 min. Discard the flow through and collection tube. Place spin filter into a new MoBio 2mL tube and load another 650 µl , centrifuge and repeat until all the supernatant has been pass through the spin filter.

(i) Place the spin filter basket into a new labeled MoBio 2mL collection tube and add 600µL of Solution PW4.

(j) Centrifuge the tubes at 13,000 x *g* for 1 min and discard flow through. Place spin filter in a new, labeled MoBio 2mL tube.

(k) Add 550µL of Solution PW5 and centrifuge at 13,000 x *g* for 1 min. Discard flow through, place spin filter into a new MoBio 2mL labeled tube and centrifuge again at 13,000 for **2 min**. Be sure all traces of EtOH are gone.

(l) Place the spin filter into a new labeled 1.7mL lab supplied MCT labeled with the sample identification number.

(m) Add 100µL of sterile water (autoclaved, de-ionized) to the center of the white filter membrane, let it sit for 1-2 minutes, then centrifuge at 13,000 x *g* for 1 min.

(n) Discard the spin filter and store the eluted DNA samples at −20°C. If more than one extraction tube was required for a single sample, combine all replicates into one final extraction tube.

(4) DNA extraction elutes should be placed into a designated freezer for overnight or longer storage, or, if used for PCR within 1-4 hours, stored in the refrigerator. Make note of sample addition to freezer log if necessary (see Exhibit 7). Note completion of extraction on sample log.

5.5.5 Qiagen DNeasy Kit (or equivalent kit) Procedure for *filters*

(1) Remove samples that have been designated to be extracted from freezer.

(2) Label one set of 1.5ml MCT for the extraction (one tube per filter). You will have time to label the rest (3 more 1.5mL lab supplied MCT, one spin filter tube, 3 Qiagen collection tubes) during the 1-hour incubation. Be sure you have added positive and negative extraction controls to each eDNA extraction procedure batch.

(3) Add 370 µL ATL to each tube.

(4) Add 30 µL protease K to each tube.

(5) Remove the appropriate filter sample and transfer to the labeled tube. Only one filter can be extracted per MCT. If more than two extractions are required for one sample, combine products into a single tube once extractions have been completed (during step 17). Be sure to push the filter down into the ATL/proteinase K mixture (use a clean pipet tip) or tap MCT on bench.

(6) Incubate at 55°C for 1 hour. During incubation, label two more MCTs, and a spin-column assembly for each sample, and set up three additional collection tubes per sample. Print labels for archived filters and extracts.

(7) Remove from incubator and centrifuge at ≥ 16,000 x *g* for 5 minutes.

(8) Transfer about 200-300 µl of supernatant, or whatever amount is easily pipetted off of the filter or swab, to a new 2 mL MCT. If the filters absorb too much supernatant as you work through the sample batch, re-spin samples another 5 minutes as needed to ensure you can easily collect supernatant without too much effort.

(9) Archive tube and filter at -80°C. Archive these until the samples have been completely processed. Once results are finalized, these can be disposed of because extracts will be permanently archived.

(10) Add 400 µL Buffer AL.

(11) Add 400 µL ethanol (96 – 100% molecular grade). Mix thoroughly by vortexing.

(12) Transfer about half of the mixture by pipet into a DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge at ≥ 6000 x *g* for 1 minute. Discard flow-through and collection tube.

(13) Transfer the remaining mixture by pipet onto the same spin column and place in a new 2 mL collection tube. Centrifuge again at ≥ 6000 x *g* for 1 minute. Discard flow-through and collection tube.

(14) Place spin column in a new 2 mL collection tube. Add 500 µL Buffer AW1. Centrifuge at ≥ 6000 x *g* for 1 minute. Discard flow-through and collection tube.

(15) Place spin column in a new 2 mL collection tube. Add 500 µL Buffer AW2. Centrifuge at 18,000 x *g* for 3 minutes. Discard flow-through and collection tube. Be careful to avoid sloshing the ethanol in the collection tube up onto the filter column. It is important to prevent ethanol from touching the filter because ethanol is a PCR inhibitor.

(16) Transfer the spin column to a new 1.5 mL or 2 mL MCT.

(17) If there are 8 or fewer filters per sample, elute the DNA by adding 200 µL Buffer AE to the center of the spin column membrane. Incubate for 1 minute at room temperature (15 - 25°C). Centrifuge at ≥ 6000 x *g* for 1 minute. If there are more than 8 filters, elute with only 100 µL Buffer AE so that all of the replicates can be pooled into one extraction tube.

(18) Discard the spin column, combine multiple extractions for a single sample if necessary. Store the eluted DNA samples at −20°C. If DNA is to be immediately used for PCR, keep on ice.

5.5.6 Qiagen DNeasy Kit (or equivalent kit) Procedure for *centrifuged* samples

(1) Label one set of 1.5-ml MCT for the extraction. You will have time to label the rest (3 lab-supplied 1.5-mL MCT, 1 Qiagen spin tube, and 3 Qiagen collection tubes) during the 1-hour incubation. Be sure you have added positive and negative extraction controls to each eDNA extraction procedure batch.

(2) Add 370 µL ATL to each tube.

(3) Add 30 µL proteinase K to each tube.

(4) Remove sterile swabs from pack and place one swab into each tube of ATL/proteinase K mix.

(5) Move dried samples in centrifuge tubes to the extraction room. Use the moistened swab to swab the bottom of each tube included in the field sample (e.g. if there are 5 tubes per sample, swab the bottom of all 5 tubes with the moistened swab). If the swab becomes covered with debris, rinse swab in ATL solution for that sample in the extraction tube and proceed swabbing the rest of the replicates. BE careful to avoid cross-contamination at this step.

(6) Place the swab back into the ATL mixture, break the wooden stick and close the tube.

(7) Incubate at 55°C for 1 hour. Label the rest of the tubes and print final archive labels for filters and extracts.

(8) Remove from incubator and centrifuge at ≥ 16,000 x *g* for 5 minutes.

(9) Transfer about 250-300 µl of supernatant to a new 2 mL centrifuge tube. If the swabs absorb too much supernatant as you work through the sample batch, re-spin samples another 5 minutes as needed to ensure you can easily collect supernatant without too much effort.

(10) Archive tube with swab at -80°C. Archive these until the samples have been completely processed. Once results are finalized, these can be disposed of because extracts will be permanently archived.

(11) Add 400 µL Buffer AL.

(12) Add 400 µL ethanol (96 – 100%, molecular grade). Mix thoroughly by vortexing.

(12) Transfer about half of the mixture by pipet into a DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge at ≥ 6000 x *g* for 1 minute. Discard flow-through and collection tube.

(13) Transfer the remaining mixture by pipet onto the same spin column and place in a new 2 mL collection tube. Centrifuge again at ≥ 6000 x *g* for 1 minute. Discard flow-through and collection tube.

(14) Place spin column in a new 2 mL collection tube. Add 500 µL Buffer AW2. Centrifuge at 18,000 x *g* for 3 minutes. Discard flow-through and collection tube.

(15) Transfer the spin column to a new 1.5 mL or 2 mL MCT.

(16) If there are 8 or fewer filters per sample, elute the DNA by adding 200 µL Buffer AE to the center of the spin column membrane. Incubate for 1 minute at room temperature (15 - 25°C). Centrifuge at ≥ 6000 x *g* for 1 minute. If there are more than 8 filters per sample, elute with 100 µL Buffer AE so that all of the replicates can be pooled at the end.

(17) Discard the spin column, combine replicates if needed, and store the eluted DNA samples at −20°C. If DNA is to be immediately used for PCR, keep on ice.

5.6 PCR Amplification of eDNA Samples

5.6.1 Purpose

In order to determine if the DNA of a specific species is present in the concentrated water samples taken in the field, the total DNA extracted from the filtered samples must be amplified using species-specific primers.

5.6.2 Source

Filters or centrifuge tubes from eDNA sampling have been received by WGL eDNA Team and DNA has been extracted. DNA elutes from samples should either be located in designated −20°C freezer or carried from the DNA extraction room to PCR room. Be sure the room is irradiated with UV light for 30 minutes prior to use. Ensure thermalcyclers are available before mixing the master mix. Master mix with or without template DNA can not sit longer than it takes to prepare the samples for cycling.

5.6.3 PCR Quality Assurance and Chain-of-custody

This stage of DNA processing is particularly susceptible to contamination and, subsequently, inaccurate results. Carefully follow quality control and COC steps listed below:

(1) PCR-type hood bench should be wiped before and after use with 10% bleach. Validated commercially available sterilization reagent such as LookOut® DNA Erase® may be preferred. PCR room should be sterilized using a built-in UV lights if available.

(2) After an item or surface is cleaned with bleach, it must be rinsed with purified water or alcohol to prevent the build-up of sodium hypochlorite crystals. Instruments or equipment cleaned with bleach should be rinsed to avoid corrosion.

(3) Centrifuges, thermal cycler, tube racks, pipettes, and any other equipment used for PCR amplification should be cleaned before and after each use.

(4) Use autoclaved, filtered, or commercially sterile molecular grade water prior to use for setting up PCR reactions.

(5) Aerosol-resistant pipette tips should be used. Place the sterile tip on the pipette immediately prior to use. If the pipette is set down with the tip on, discard the tip. A new pipette tip must be used for the addition of each reagent to a sample tube.

(6) Use autoclaved sample tubes for PCR master mix.

(7) Close each tube immediately after labeling and after the addition of sample or reagents to prevent cross-contamination.

(8) Be sure to only touch the tip of the MCT cap or use a tube opener, clean Kimwipe®, or other suitable barrierto open MCT.

(9) Record all solution batch numbers used for reactions in lab notes.

(10) PCR reagents should be aliquoted (a portion of the original stock) to avoid excessive freeze-thawing and to protect stock reagents if contamination occurs.

(11) Lightly vortex (quick touch, because vigorous vortexing can damage *Taq*) to mix sample and quick-spin/centrifuge tubes before opening the reagents to avoid splashes or drips from cap when opening. Uncap and close tubes carefully to prevent aerosol contamination.

(12) Any revisions to the DNA amplification protocol must be approved by the eDNA Project Leader and documented in writing.

Cautions: Wear powder-free latex or nitrile gloves throughout the DNA amplification and gel electrophoresis procedures. Ethidium bromide, used in DNA gel electrophoresis to visualize DNA, is a known mutagen that affects biological processes.

5.6.4 Procedure

(1) If DNA samples (extraction elutes) are removed from freezer, note on freezer/ambient log (see Exhibits 7-9). Also note on sample log (Exhibit 6).

(2) Use preprinted 96-well plate map (Exhibits 12-13) or build plate map in LIMS to determine which samples will be pipetted into which wells. Clearly mark plate identification on bottom edge skirt of plate. Write plate identification information (e.g. FY_case#_sample numbers_species_initials_date) in lab notes Legible labels for use while working must be complete to allow for easy down-stream processing in the sequencing lab.

(3) Make sure sample map for each plate is entered into the LIMS or attached to lab notes and a signature is written across the map and lab book page.

(4) Follow DNA amplification protocol detailed below.

Primers specific to either *Hypophthalmichthys molitrix* (Silver Carp) or *H. nobilis* (Bighead Carp) are used to screen eDNA samples and amplify unique DNA sequences in each species potentially present in the eDNA samples by PCR. The PCR programs used to amplify the extracted DNA are specific to the primer set used. The PCR protocol has been optimized to utilize Platinum® *Taq* DNA polymerase (Invitrogen Corporation, Carlsbad, CA) in the eDNA screening. If other brands of *Taq* are used, optimization of the recipe and thermal profiles must be executed. Eight reactions are set up for each sample, in addition to negative (DNA blank) and positive (DNA extracted from tissue[2]) controls for each master mix. The PCR reactions are prepared as follows:

(1) Wipe lab bench area with 10% bleach, 75% Ethanol, or commercial DNA sterilization wipes. Also wipe down work area with PCR hood. Use built-in UV lamps to radiate clean room for 30 min prior to PCR set-up.

(2) Electronic pipettors should be wiped down with one of the solutions or wipes listed in Step 1.

- In the clean reagent room, obtain all PCR master mix reagents (using only those that have not expired and that have been tested and found viable).
- 10X PCR buffer (comes in *Taq* kit)
- 10mM equally mixed dNTP solution (2.5 mM per nucleotide)
- 50 mM Mg2+ solution (concentration in Platinum *Taq* kit; use what comes with your brand)
- Species-appropriate forward primer

[2] Extract from tissue using commercial DNA extraction kit (e.g., Qiagen DNeasy Blood & Tissue Kit), and manufacturer protocol. Run test PCR before relying on any DNA extract for eDNA assay positive controls.

- Species-appropriate reverse primer
- *Taq* DNA polymerase (we use Platinum, but any hot-start *Taq* can be used)
- Sterile molecular grade water (commercially sterile or Millipore filtered, autoclaved)

Allow reagents to thaw. Do not vortex primers or *Taq* too violently.

(3) Record in lab notebook the lot number of all reagents used.

(4) Prepare PCR master mixes in clean reagent room. The master mix volume can be adjusted according to the number of samples to be processed. In order to make sure that master mix does not run out prior to supplying all the desired reactions (this may occur as a result of minor errors or variations in pipetting volumes), it is generally helpful to make more than enough master mix than is needed for the desired number of reactions. For example, make enough master mix for 100 reactions when actually preparing for 96 reactions. NOTE: If positive extraction controls consist of a different species of DNA, be sure to make a small separate master mix for those samples and use primers specific to the content of the control sample. Negative extraction controls should be amplified with the Silver or Bighead Carp master mix.

(a) Each Initial PCR 1X reaction should contain:
- 2.5 µL 10X PCR buffer
- 0.5 µL dNTP (10 mM mixed dNTP)
- 0.75 µL Mg2+ solution (50mM)
- 0.5 µL forward primer (10µM working dilution)
- 0.5 µL reverse primer (10µM working dilution)
- 0.25 µL Platinum® *Taq* polymerase (= 1.25 U)
- 19.0 µL sterile water.

Move prepared mix from reagent room into PCR room.

(5) Remove DNA extracts from freezer or fridge (fill out logs as needed), vortex (quick touch) and quick-spin down the extract tubes. Take them into the PCR room. Place the 96-well PCR plate onto a clean surface, positioned from left to right.

(6) Fill the plate wells with 24 µl PCR mix. Carefully pipette 1 µL of each sample to be screened into each well of a column, changing the pipette tip between each sample. Each column of eight wells should be filled with the same sample (i.e., eight replicates per sample to be tested). The first 11 columns of the PCR plate can test 11 different samples for one target species. Into the 12th column, pipette 1 µL of sterile water into the bottom three wells (F, G, H) and pipette 1µL of each of the target species positive control DNA into each of the top four wells (A, B, C, D). Leave the intervening well (E) empty.

(7) Place the positive control DNA back in to the appropriate −20°C freezer and change gloves immediately in order to reduce risk of contamination.

(8) Place PCR film over the PCR plate and press firmly (or use an automatic plate sealer) to ensure the edges of all wells are sealed. Gently tap a few times on the lab bench to ensure thorough mixing of each reaction. Spin down the plate in the plate spinner to ensure all DNA is down in the master mix.

(9) Place the 96-well PCR plate in the thermal cycler, close and secure lid, and select the appropriate PCR thermal program (thermal cycle programs for Silver Carp and Bighead Carp utilize different

annealing temperatures). The thermal programs for the current eDNA markers (Jerde et al. 2011) both consist of:

- Initial denaturation at 94°C for 10 min

Followed by 45 cycles of:
- 94°C for 1 min,
- 50°C for Silver Carp program or 52°C for Bighead Carp for 1 min
- 72°C for 1.5 min.

Followed by:
- final extension at 72°C for 7 min
- 4°C hold temperature until plate removed from thermal cycler.

(10) Record the plate ID, thermal cycler unit or head, plate orientation, and run times for the PCR plate in the PCR log (Exhibit 10).

(11) Place cycled PCR plates and product in designated −20°C freezer for long-term (more than overnight) storage, in the designated 4°C refrigerator for short- or mid-term storage (1–12 hours). If you leave for the night, the thermal cyclers are set to hold at 4^0C forever. Remove plates promptly in the morning.

(12) Under no circumstances should you open or uncover PCR plates that have been cycled in the PCR room. Only open cycled plates in the post-PCR rooms.

5.7 Gel Electrophoresis of eDNA PCR Assays

5.7.1 Purpose

Once amplified, the DNA samples should then be subjected to gel electrophoresis in order to visualize the amplified DNA. This method is useful in determining the presence of DNA from the target species in different aquatic environments.

5.7.2 Source

PCR product following amplification can be taken either from cold storage (see #11 above) or directly from the thermal cycler.

5.7.3 Gel Electrophoresis Assurance and Chain-of-custody

This stage of DNA processing is particularly susceptible to pipetting error. It is also highly susceptible to mislabeling and, consequently, confounding of sample results.

(1) Draw or otherwise produce a map of which sample will be electrophoresed on which gel and in which lane of the select gel.

(2) Carefully pipette samples so as to avoid:
- Injecting samples to incorrect wells.
- Piercing the bottom of sample wells and losing PCR product.
- Spill over from adjacent wells.

(3) Record all solution batch numbers or name/date identification for stock solutions. Record precast gel batch identification number if appropriate.

(4) Centrifuge plates, strip tubes, etc. before removing film or caps in order to prevent aerosol cross-contamination.

(5) Any revisions to the DNA amplification protocol must be approved by the eDNA Project Leader (and documented) and incorporated into a revised QAPP.

5.7.4 Option A Procedure

(1) Prepare 2% agarose gels with SB (sodium hydroxide and boric acid) buffer and allow the gel to polymerize for a minimum of 25–30 min prior to loading samples. Gels can be prepared at any time prior to PCR or immediately after PCR. However, once you begin loading a gel finish loading and run the gel immediately.

(2) To prepare PCR samples for gel electrophoresis, transfer 10µl from each well of the 96-well plate to new wells in an identically labeled 96-well plate. Add 2µl of loading dye (see recipe below) to each well with PCR product (loading dye = 500µl 6X loading dye, 500 µl DMSO, 1µl SYBR Green I)

(3) Place the 2% agarose gel in the electrophoresis chamber that contains SB Buffer and remove the gel combs. In the first well of each row on the agarose gel, load 100bp DNA ladder/loading dye mix. Next, load 10µL of each sample mixture (i.e., each PCR reaction and loading dye), and negative controls into the remaining wells. Be sure 10 µl of a positive control PCR product is in the last lane of each row. Run electrophoresis at ~250V for ~45 min depending on migration times through the gel. Times and voltages required to run each gel are approximate (different buffers and higher content agarose gels will require different run times and voltages, use what works in your lab).

(4) Be sure to annotate the gel loading map in the notebook as you observe the gel in the geldoc. Interpret positive samples and note successful positive and negative controls on each gel. After documentation (5.8), dispose of gel in the garbage.

5.7.5 Option B Procedure

Pre-cast 2% E-Gel® 48 gels (Invitrogen) are used with the E-Base™ system (Invitrogen).

(1) Open E-gels and load into the bases.
- Remove gel from the pouch. Remove comb from the gel.
- Slide gel into the two electrode connections on the Mother E-Base™ or Daughter E-Base™. If gel is properly inserted, a fan in the base begins to run, a red light illuminates, and digital display shows 20 min.
- Load each gel within 30 min of removing gel from the pouch and run within 15 min of loading.

- Selecting Program on E-Base™
- Connect the Daughter E-Base™ to a Mother E-Base™ or another Daughter E-Base™ if running multiple gels.
- Select program EG by pressing and releasing the pwr/prg (power/program) button on the base.

- Loading E-Gel® 48 Gels
- Load 15µL PCR product into each well of an E-Gel® 48 gel. Keep all sample volumes uniform. Load with a multichannel pipettor.

- Load appropriate DNA ladder (distinct bands between 0-500 bp) into far left well (labeled M) and positive control PCR product into the far right marker well. Ensure the marker salt concentration is similar to that of adjacent samples (2% gel uses 100 bp DNA Ladder).
- Load 15µL of sample buffer containing the same salt concentration as the sample, or sterile water, into any empty wells.

(2) Run Conditions

- To begin electrophoresis, press and release the pwr/prg button on the Mother E-Base™ and Daughter E-Base™. The red light changes to green.
- At the end of the run (signaled with a flashing red light and rapid beeping), press and release the pwr/prg button on the base to stop the beeping and flashing red light.
- Remove gel cassette from the base and analyze results.

Note: if a gel is removed before a run is complete, a gel must be replaced and the unit allowed to run out until the timer counts down to zero. There is no option to reset the base.

5.8 eDNA Gel Documentation and Storage

5.8.1 Purpose

Once eDNA gels have been visualized, the results must be documented, interpreted (i.e., scored), and recorded. In some cases, very light bands may be visible, making scoring difficult. Documentation, labeling and saving for sequence confirmation, and storage are critical for later quality control review.

5.8.2 Source

Following electrophoresis, agarose gels should be immediately documented. Following documentation, PCR products requiring sequencing must be labeled and organized for sequencing.

5.8.3 Gel Documentation and Storage Assurance and Chain-of-custody

Because of the difficult nature of scoring some results, careful records must be kept of all gels and results. These results must be maintained so as to minimize the risk of tampering or data loss.

(1) Gel image quality must be assessed at the time images are obtained. Images should exhibit all bands on gels as clearly as possible, if this is not possible, it must be noted on the gel data sheet (Exhibit 14). All gel digital image files should be saved and should be archived at the end of each working day. All gel image data are referenced to the samples case number to make sure the consistency of the sample custody.

(2) Gel score data should be entered and stored in the appropriate database in an Excel file and on the data sheet in the laboratory notebook, or in LIMS.

(3) All reports should reviewed by the eDNA Processing Leader before being reported.

(4) A paper copy of the report should be held in the files for 5 years.

(5) Electronic copies of all reports should be held for 5 years or longer, as space permits.

(6) Any substantive revisions to the DNA amplification protocol must be approved by the eDNA Processing Leader and approved by the eDNA Coordinator. Any such changes must be incorporated into a revised QAPP.

5.8.4 Procedure

(1) After electrophoresis is complete, remove casting tray with gel from the electrophoresis chamber and place the gel onto the gel scanner (BioRad Molecular imager FX), select DNA ethidium bromide stain gel, set up scanning area, and then select 100 micrometer to start scanning the gel.

(2) Alternatively, place the gel on a UV transilluminator equipped with a digital camera, such as the Alpha red Imager (Cell Biosciences, Inc.), and capture a digital photograph of the gel.

(3) After the gel scanning is done, properly label file name and save the file on the hard drive immediately.

(4) If not using LIMS, print out a picture of the gel image and insert into lab book. A copy should be kept with the Project Lab Book. Be sure to sign across the print out and the lab book page.

5.9 Gel Interpretation

5.9.1 Purpose

Once a gel is visualized, the quality of the results and presence of potential positive bands must be assessed in order to determine which samples need to be further assayed.

5.9.2 Source

Immediately following the cessation of electrophoresis, the agarose gel containing the eDNA PCR products should have been visualized on either the UV-based imager or the laser-based imager. In both cases, the gel image should be captured (saved to hard disk) immediately and then printed.

5.9.3 Gel Interpretation Quality Assurance and Chain-of-custody

(1) The positive controls should have bright bands at the appropriate migration distance (number of base pairs), indicating a positive reaction.

(2) No bands at the targeted sizes (~200bp silver, ~300bp bighead) should be observed in the negative controls.

(3) If any of the initial PCR reactions are positive (i.e., a visible band at the appropriate migration distance), the initial sample is designated a “presumptive positive”.

(4) Record the number of presumptive positive reactions for each sample both in the gel electrophoresis lab notebook and in the excel file on the lab computer.

(5) Presumptive positive results will initiate a series of results confirmation mechanisms (see below). These mechanisms include screening the transport and equipment controls, and DNA sequencing.

(6) Once presumptive positives are documented:
   a. Fill in the quality control results in the eDNA sample log (Exhibit 6).
   b. Notifiy the eDNA Processing leader.
   c. Color in the plate seal above the wells containing presumptive positive samples on the PCR product plate. Move all plates requiring sequence confirmation into the freezer in the sequencing room. Plates without positives can be discarded.

5.10 Confirmation of Positive Results

5.10.1 Purpose

These confirmation mechanisms are initiated if a sample returns as a positive for the PCR test (any number of the eight reactions, e.g., one of eight up to eight of the eight PCR reactions).

5.10.2 Source

Positive results for Asian carp eDNA require that those positive samples be further assayed. The original DNA elutes from samples should be located in designated −20°C freezer.

5.10.3 Gel Interpretation Assurance and Chain-of-custody

Presumptive positive assays (PCR reactions) are validated through DNA sequencing and testing of additional control samples.

Any revisions to the DNA QA/QC amplification protocol must be approved by the eDNA Processing Leader and approved by the assigned USFWS senior executive. Any such changes should be incorporated into a revised QAPP.

5.10.4 Procedure

(1) Conduct PCR assays of the paired equipment control for each presumptive positive. DNA extraction, amplification, documentation, and interpretation following protocols detailed above (Sections 5.6–5.9).

(2) Ensure that the transport blanks (see 2.2.2 (6) and 2.3.2 (10)) have been tested for that sample group (i.e., from the same cooler in which the presumptive positive sample was transported).

(3) For all presumptive positive samples, bidirectional sequencing confirmation is performed. This is done by using a commercially available gel extraction kit (e.g., Qiagen Qiaquick Gel Extraction kit) per the manufacturer's recommendations on the positive PCR reactions, or E-Gel® CloneWell Agarose Gels (Invitrogen) per manufacturer's recommendation.

(4) If the equipment control and transport blanks test negative, the sample is designated a "**confirmed positive**."

(5) The following sequencing reaction can be done either by cloning (TA TOPO cloning kit used for sequencing per manufacturer's recommendation) then sequencing, or by a direct Sanger sequencing method (ABI BigDye® Terminator v3.1 or v1.1 Cycle Sequencing Kit) modified by WGL. BigDye Terminator Reaction Master Mix for 1X reaction:

- 1 µL BigDye terminator mix,
- 4 µL 5X reaction buffer,
- 0.8 µL either SC/BH forward primer, and
- 10.2 µL of water.

Add 16 µL of master mix to the 4 µL of purified DNA. Total reaction size is 20 µL.
The positive control reaction of sequencing was done per manufacturer's recommendation.

For each pGEM control PCR master mix:

- 1 µL BigDye terminator mix,
- 4 µL 5X reaction buffer,
- 2 µL m13 primer,

- 8 µL water, and
- 2 µL pGEM.

Add 20 µL to each control well.

Place the PCR plate on the thermal cycler and begin temperature cycling protocol. Program the thermal cycler as follows: 25 cycles of [96°C for 10 sec, 50°C for 10 sec, 60°C for 4 min], then ramp to 4°C.

(6) Option 1: To clean the BigDye reaction with EDTA precipitation:

- Spin plate after removing from thermal cycler (just to make sure that everything is at the bottom of the well).
- Add 5 µL of 125mM EDTA to each well.
- Add 60 µL of 100% EtOH.
- Seal the plate and mix by inverting four times.
- Incubate at room temperature (RT) for 15 min.
- Spin plate at 3000xg for 30 min (at 4°C) or at 2000 x g for 45 min. Proceed to next step **immediately**.
- Invert the plate and spin at 185 x g for 1 min (time from when rotor starts).
- Add 60 µL of 60% EtOH.
- Spin at 1650 x g for 15 min at 4°C.
- Invert plate and spin at 185 x g for 1 min.
- Resuspend samples in 20 µL Hi-Dye.

Option 2: To clean the BigDye reaction with Sephadex in Centri-SEP spin columns or Millipore plates, use columns for 1-16 samples and plates for 17 or more.

Centri-SEP column procedure

Materials list:

Spin columns (preloaded with dry sephadex, caps, and bottom caps), one per sample
Pipets and tips
Centrifuge with MCT rotor
Reagent grade water
Labeled 1.5-mL MCT and tube rack
Vortexer

(1) Column Hydration

a. Tap column on counter to settle sephadex gel into the bottom.
b. Remove cap and add 800 µl sterile ddH_2O. Replace cap, invert, and mix well on vortexer, ensuring all gel is hydrated. Invert again, vortex and place in rack.
c. Let sit 30 minutes at room temp in a tube rack.
d. While columns sit, label 1.5ml MCT with sample names for collecting cleaned product.

(2) Remove excess water

a. Check each tube for bubbles, if there are bubbles in the gel, invert the tubes and vortex so all the slurry moves toward the cap.
b. Invert and gently place in tube rack, allowing gel to settle.
c. Remove caps, then bottom, and place column in collection tube to drain.
d. Allow to drain until about 200-250 µl water has drained. If needed, gently apply pressure with a gloved finger to get the water draining.
e. Discard fluid, replace column in collection tube.
f. Place columns into centrifuge, making sure the indicator tabs are up.
g. Spin at 750xg(rcf) for 2 min
h. Discard the collection tube, blot any water off the bottom of columns and place column into labeled sample collection tube.

(3) Sample processing

a. Hold the column up to eye level, and carefully place all of the cycle sequencing product onto the center of the packed column, being careful to not disturb the column. Don't touch the side of the column with the product or the pipet tip.
b. Place collection tube into centrifuge with caps open and facing inward and the indicator tab of the column facing up.
c. Spin at 750xg(rcf) for 2 min. Discard spin column.
d. If you have more than 32 samples, you must dry filtrates in the spin vac at ~60* for ~45minutes. Once dry, you may freeze for up to two weeks or go directly to analyzing on the sequencer. If you have 32 samples or less, you may run those directly on the sequencer in the elution water.

Millipore plate sephadex procedure

Materials list:

Millipore filter plate with centrifuge alignment frame
Plain 96-well plate to catch waste water (can reuse the same one)
Sequencing plate (with bar code)
Sephadex G50-fine

Millipore column loader and tray
Centrifuge with plate rotor
Reagent grade water

(1) Prepare sephadex plate:
   a. Pour dry sephadex into the black metal loading plate which is in the plastic catchment tray.
   b. Use the clear squeegee to level each well you need for cleanup (tape off unused wells).
   c. Scrape excess powder into the catchment tray so you don't waste too much sephadex.
   d. Invert a multiscreen plate over the metal tray and hold them together while you invert the tray to fill the wells with sephadex. Make sure you are filling the wells you wish to use! Tap gently to make sure all of the sephadex moves into the filter plate.
   e. Pour excess sephadex from the tray back into the sephadex container and cap tightly.
   f. Add 300 µl of water to each well of the plate and let sit *3 hours at room temp*. (these plates can be prepared ahead of time, wrapped tightly in plastic wrap, placed in a Tupperware with a moist towel and refrigerated for two weeks.)

(2) Remove excess water and pack columns prior to use:
   a. Place the filter plate over a plain 96-well plastic plate, and load into the plate rotor. Don't use the interior lid because it does not fit over stacked plates.
   b. Centrifuge at 910xg(rcf) for 5 min.
   c. Dump the waste water into the sink and rinse catchment plate (set aside for another use).

(3) Clean sequencing reactions with the columns.
   a. Use the multichannel pipet to carefully add reactions to the *center* of each column.
   b. Place the filter plate over a 96-well sequencer plate making sure wells A1 are aligned.
   c. Centrifuge at 910xg(rcf) for 5 minutes.
   d. Dry filtrates in the spin vac at ~60^{0}C for ~20 minutes. You may seal plate and freeze for up to two weeks or go directly to analyzing on the sequencer. If you have 32 samples or less, you may run those directly on the sequencer in the elution water.
   e. Once samples are dry, add 15 µl HiDi to each sample. Samples in Hi-Dye can be stored at 4°C overnight, but may not be left any longer than 12 hours.

(4) To sequence:
   - Denature samples with HiDi for 5 min at 95°C in thermocycler (do not denature samples run wet)
   - Place immediately on ice
   - Load into sequencer plate and onto sequencer.

Resulting sequencing reactions that are successful are screened in GenBank (http://www.ncbi.nlm.nih.gov/blast) using the BLAST (Basic Local Alignment Search Tool) algorithm. If the resulting sequence is a positive match to the targeted species of Asian carp, the sample is designated a "**confirmed positive – sequenced**".

References: User Manual, "BigDye® Terminator v3.1 Cycle Sequencing Kit"
http://www.ibt.lt/sc/files/BDTv3.1_Protocol_04337035.pdf
"TOPO TA Cloning® Kit for Sequencing" http://tools.invitrogen.com/content/sfs/manuals/topotaseq_man.pdf
"QIAquick Gel Purification Kit"
http://molecool.wustl.edu/krolllab/Kroll_Lab_Protocols/Molecular%20Biology%20protocols/Cloning%20protocols%20folder/Gel%20extraction-Qiagen.pdf

5.11 Communication of eDNA Assay Results and field data from WGL to USFWS Midwest Region Leadership

5.11.1 Purpose

To convey to Serive designated personnel the progress and results of eDNA assays.

5.11.2 Source

WGL keeps a record of each samples progress through the eDNA assay procedure. These records are summarized for each batch for reporting to the eDNA Coordinator in USFWS Midwest Region.

Each field sampling agency keeps a record of field data. These records are summarized and reported to the eDNA Coordinator in USFWS Midwest Region via the eDNA database.

The eDNA Coordinator in USFWS Midwest Region collates field and lab data for reporting results to partners and the public.

5.11.3 Quality Control

The WGL eDNA Processing Leader will provide updates and reports to the USFWS Midwest Region eDNA Coordinator. At this time, Emy Monroe is the WGL eDNA Processing Leader and Kelly Baerwaldt is the USFWS Midwest Region eDNA Coordinator. Any permanent or temporary changes to either position should be communicated immediately to the assigned USFWS senior executive. Any permanent changes should be incorporated into a revised QAPP.

Any revisions to the reporting procedures must be approved by the eDNA Processing Leader and approved by the assigned USFWS senior executive. Any such changes should be incorporated into a revised QAPP.

5.11.4 General Procedure (for details see Appendix E for SOP)

The USFWS eDNA Processing Leader should, on every Friday during the period over which the WGL eDNA Team is processing samples, provide updates on all sample batches to the USFWS eDNA Coordinator. Reports should be organized by case and should consist of spreadsheets showing the stage of processing for each sample. Additionally, following approval by the eDNA Processing Leader, the WGL PRR should convey final results for each case (all samples confirmed as positive or negative for AC eDNA) within 24 hours of completion of processing for the last sample within a case. In addition, weekly updates on general lab processing progress (without results) will be send to FWCO Project Leaders.

The USFWS eDNA Coordinator may request updates from the eDNA processing leader at any point. The WGL eDNA Processing Leader is expected to respond as soon as possible during normal working hours.

Table 5-1. List of critical components for eDNA Processing, and component information for ERDC. No vendor is listed for generally available supplies.

Equipment
Enough sets of manual pipettes ranging from 0.1µl to 1000µl so that each designated area has its own set Electronic 8 or 12 channel multi-dispense pipettes ranging from 0.5µl to 125µl ERDC: Finpipettes models # (0.1-25 µl)
Programmable thermal cyclers equipped with four 96-well plate heads • Thermal cycler should be capable of self-test upon instrument startup. • All thermal cyclers and heads should be equipped with the heated lid function.
Centrifuge equipped with programmable speeds and time. • Interchangeable Rotors capable of holding 96-well plates and tubes of various sizes (5mL, 1.5mL, and 2mL).
Electrophoresis Chambers including gel casts and gel combs
Sterile Hoods equipped with UV light and Hepa filters
Autoclave
Locking Refrigerator monitored at 4˚C equipped with a temperature sensitive alarm
Locking Freezer monitored at −20˚C equipped with a temperature sensitive alarm
Locking Freezer monitored at −80˚C equipped with a temperature sensitive alarm
Sequencer
Gel Image Scanner
UV Stratalinker
Reagents
LookOut DNA Erase (Sigma Aldrich Cat# L8917 refill cat # L9042)
Power Water DNA Isolation Kit (Mo Bio Laboratories Cat # 14900-100-NF)
QIAquick Gel Extraction Kit (Qiagen Cat# 28706)
Taq DNA Polymerase (Platinum taq Life Technologies Cat# 10966083)
Big Dye Terminator Sequencing Kit v3.1 (Applied Biosystem Cat # 4337455)
Nuclease Free Water (Ambion Cat # AM9932) (Autoclaved before use.)
pGEM-3Z(+) Vector 20 g (Promega Cat # P2271)
Ethanol (Sigma Aldrich Cat # E7023)
dNTP
MgCl2
10X buffer
Primers (forward and reverse)
20X SB buffer
Cloning Reagents
Sequencing Reagents
Agarose
Bleach Solution (10%)
Ethidium Bromide Gel Stain
100 bp DNA ladder
Gel Loading Dye
Disposable Supplies
Sterile Pipette Tips: various sizes and types to fit manual and electronic pipettes.
Sterile Nuclease Free conical tubes: 1.5 mL, 2.0 mL, 5.0 mL
Sterile 96-well PCR Plates – UV cross-linked prior to use.
Gloves: Nitrile or Latex, various sizes
Forceps: cleaned by soaking a minimum of 10 min in a 10% bleach solution or by LookOut DNA Erase following manufacturer protocol.
Sterile seals for 96 well plates.
Sterile Optical Sealing Tape for 96 well plates.

Kimwipes®
Paper Towels
Countertop moisture barrier papers
Black Permanent Markers

SECTION 6

6. INTERNAL QUALITY CONTROL CHECKS

Details on quality control are found within each of the various protocol sections. In summary, however, quality control relative to sample contamination is covered by the transport (or cooler), equipment, DNA extraction, PCR, and sequencing blanks or negative controls. Quality control for efficacy of methodology, solutions, etc. is covered by positive controls for each sample handling step (extraction, PCR, and sequencing) of the eDNA protocol. Furthermore, each new solution or kit to be used in eDNA processing will be tested with positive and negative controls before or during the first use.

The WGL eDNA processing facility and protocols was reviewed by an ERDC audit shortly after completion of the transition plan and full deployment of Asian carp eDNA dedicated equipment.

6.1 Laboratory Quality Control Evaluation Criteria

Quality control is measured in two ways:

- If transport, filtering, centrifuge, hood, extraction, PCR, and DNA sequencing negative controls show product (e.g., bands in PCR or DNA sequence), the associated positive data are negated and, when possible, samples are reprocessed. Contamination of DNA extract will require that any positive samples be removed from consideration. If all other samples are negative, contamination was only an issue in the controls, and negative results may be reported.
- Positive controls are currently employed for extraction, PCR, and sequencing. If the positive controls fail to behave as expected, any sample showing an apparent lack of results will be rerun at the same time or following rerunning of the positive controls. This will be done until all positive controls produce the expected results.
- We incorporate two types of positive controls during sequencing. One positive control PCR products from positive control reactions and one sequencing reaction per plate with a standard DNA sequencing template (pGem) provided by the manufacturer. If less than a full plate are sequenced, one pGEM per batch of sequencing master mix made, and a minimum of one per plate eDNA samples that are sequenced. In the case that any of these fail, any samples that fail to produce sequence data that were run at the same time will be rerun at the same time as positive controls are rerun.
- In cases where fewer than 16 eDNA samples are sequenced, both types of positive sequence controls are still run.

SECTION 7

7. SPECIFIC ROUTINE PROCEDURES TO ASSESS DATA PRECISION, ACCURACY, AND COMPLETENESS

7.1 Field Methods and Laboratory Data

Every 3 months, a dilution series of Asian carp water will be processed starting with the filtering and/or centrifuging, and then extraction through sequencing to ensure that current practice, instruments, and personnel are maintaining the same level of sensitivity and accuracy. A brief report will be provided by the DNA Processing Lead to the eDNA Coordinator. Asian carp water will be either collected from the Asian carp holding tanks at the USGS Upper Midwest Environmental Sciences Center (UMESC), or made by collecting slime and feces from Asian carp at UMESC, or made with Asian carp cell lines from the USFWS Fish Health Center Virology lab.

SECTION 8

8. CORRECTIVE ACTIONS

Corrective actions may be required for two classes of problems: analytical/equipment problems and noncompliance problems. Analytical and equipment-related problems may develop during sampling and sample handling, sample preparation, laboratory instrumental analysis, and data review. Noncompliance issues arise when eDNA sampling, processing or lab procedure execution deviates from procedures described in the QAPP.

In the case of analytical/equipment problems or deviations from set procedures (as outlined in QAPP), the responsible lead will determine if the problem or deviation will impact the accuracy of the resulting data. If it is determined that the problem or deviation does impact data accuracy, two courses of action may be followed:

(1) If possible, the procedure is repeated until it is performed without any problem or deviation, or

(2) The sample or samples are removed and not processed any further.

In either case, a corrective action report must be completed (Exhibit 16). Careful notes of any corrective actions and what incident led to them, as well as the resolution or preventative measure(s) identified will be carefully noted in the corrective action report, which must be provided electronically to all Leaders (Project Leader, Sampling Leader, etc) as an after action report. The paper copy of the corrective action report will be maintained in the project file as a long-term record.

In the case that the responsible lead determines that data accuracy is not affected by the analytical/equipment problem or deviation from procedure, the sample or samples may continue to be processed. The responsible lead will make careful note of the incident in project records and include the rationale for continuing processing.

SECTION 9

9. PREVENTATIVE MAINTENANCE PROCEDURES

9.1 Field Equipment/Instruments

Hand-held or console installed sonar: Batteries will be changed at least once a month in hand-held units (if not required sooner) to ensure accurate readings of the instrument. In addition, reading accuracy should be checked prior to sampling season for all units. Depth readings may be checked by filling a container of a known depth with water and submerging the instrumentation. Temperature readings of the sonar may be checked against a thermometer. These records should be kept in ink in a bound notebook, where the originals are kept on site in a secure location and copies are sent to the eDNA Coordinator at the end of each sampling season.

GPS equipment: Batteries will be changed at least once a month (if not required sooner) to ensure accurate readings of the instrument. In addition, coordinate accuracy will be checked against known benchmarks.

DI equipment in the Trailer: Keep a bound hard-copy notebook in the trailer to record the total dissolved solids present each day the trailer is used. It should read zero.

Plastic 2L sample bottles: After bleaching and autoclaving, bottles will be inspected for dents and/or warping of the material. Any bottle failing inspection will be disposed of and replaced.

Forceps: Forceps will be inspected monthly, and those exhibiting large amounts of rust will be disposed of and replaced.

Carboys: Carboys will be inspected monthly for cracks in the glass that could pose a safety hazard to filtering personnel. Any carboy failing inspection will be disposed of and replaced.

Plastic tubing: Plastic tubing used to connect the carboy to the manifold will be inspected monthly for cracks in the plastic. Any plastic tubing failing inspection will be disposed of and replaced.

All other laboratory equipment will be inspected monthly and undergo proper maintenance to maintain their ideal working condition. Any equipment not performing accurately or to established standards will be disposed of and replaced.

9.2 Laboratory Instruments

Pipettes: Annually all pipettes will be inspected, calibrated, and certified. Any pipette failing inspection and certification will be disposed of and replaced.

Any thermal-cycler head that fails the manufacturers self-test upon instrument startup will be removed and replaced with the manufacturer's certified replacement part.

Equipment maintenance contracts, with annual maintenance check-ups, will be used for any appropriate equipment (i.e., DNA sequencer).

SECTION 10

10. PERFORMANCE AND SYSTEM AUDITS

10.1 Field Audits

Internal audits of field crew performance and quality controls for sampling and filtering will be made semi-annually by the FWCO field staff to make sure that all procedures in the sample collection portions of the QAPP are being followed. On sampling trips where more than two FWCO offices are on the team, the visiting FWCO eDNA leader will serve as the sampling auditor. A brief report will be made to the eDNA Program Coordinator of audit findings, including a signed checklist of audited procedures (Exhibit 16) If there are no trips with coordinating offices, then the representative on the eTeam for USFWS Midwest Region may serve as the auditor for their office.

10.2 Laboratory Audits

Internal audits of WGL laboratory performance and quality controls will be made semi-annually by the DNA Processing Lead to make sure that all procedures in the DNA processing portions of the QAPP are being followed. A brief report will be made to the eDNA Program Coordinator of audit findings, including a signed checklist of audited procedures. Participation in validation studies by the lab may suffice as internal audits, and reports of such studies may be substituted and filed with the eDNA Program Coordinator. Every 2 years an external review of WGL eDNA processing will be undertaken. The review panel or consultant(s) will be selected by the Project Lead. The DNA Processing Lead may assist the Program Coordinator in identifying one or more potential reviewers.

SECTION 11: EXHIBITS

Field Collection Summary

Case Number _____ **Sample Date** _____

Sampling Lead _____**Sampling QA/QC**_____ **Data Recorder** _____

Location_____

Samples (range) _____Blanks (CI)_____

Boat Ramp (lat, long) _____

Water Collection Personnel

Boat Operator _____

Other Personnel _____

Prep List (Initial and Date)

Bottles Cleaned _____

Coolers bleached _____

Bottles loaded _____

Blanks loaded _____

Boat cleaned _____

Boat cleaned _____

Water Processing Personnel

FWS Lead _____ FWS QA/QC_____

Other Personnel _____

Time Frame

Start _____ End_____

Bottles Iced _____

Return to Lab/Filtering Trailer _____

Other
Notes:_____

_____

Map Attached

Coolers

___Labeled bottles
___Cooler blanks

Clipboard

___pencils/pens
___Data sheets
___GPS
___Extra batteries for GPS
___Maps

Drybag

___Gloves
___Depth/temp device
___Wet Wipes
___Sunblock

Drinking water

Others

___bleach
___mop
___bucket
___camera
___Ipass
___Life jackets
___Filed float plan
___sunglasses

Field Collection Data Sheet

Exhibit 2

CASE NUMBER_____DATE_____ NAME_____ START TIME_____ Volume: 2L or 5x50ml SHEET ___of___

Sample ID	Wind Direction	Way-point	Latitude (dec.degrees)	Longitude (dec.degrees)	Temp (C/F)	Depth (m/ft)	Blank (B)	Habitat (LDB/MC/RDB /BAY/TRB)	Collect time	Processing time	Processor Initials

Notes/comments:

Chain-of-custody Form

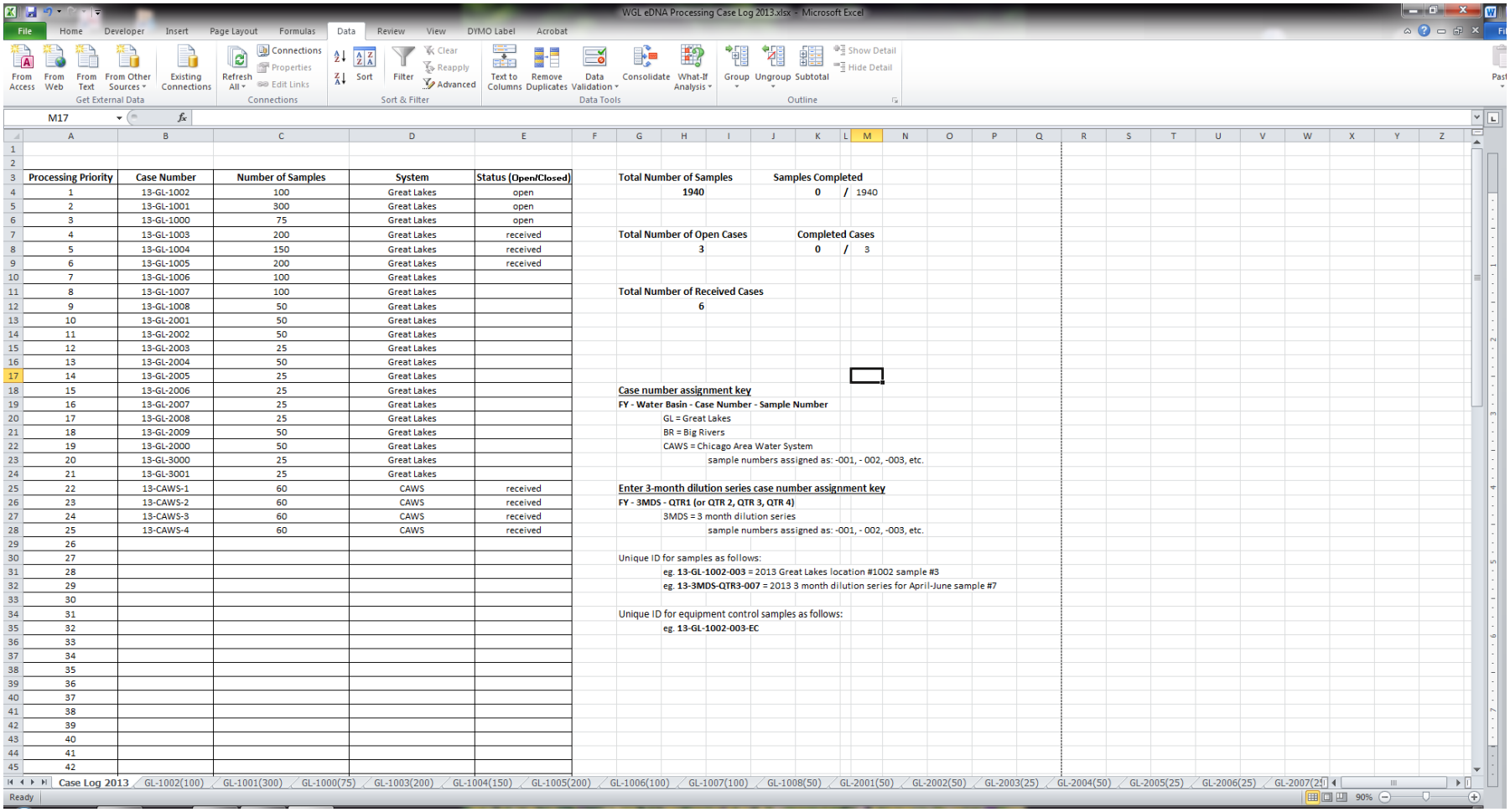
US Fish & Wildlife Service	**CHAIN-OF-CUSTODY RECORD**			
Date and time of collection:		**Collected by (first and last names):**		
Notes:				
Case Number:		**Cooler Temperature Before shipment:** **Upon receipt at Lab:**		
Sample Numbers	**From: (Print name, Agency)**	**Release signature:**	**Release Date:**	**Delivered via:** ☐ **FedEx** ☐ **In Person** ☐ **Other:**
	To: (Print name, Agency)	**Receipt signature:**	**Receipt Date:**	
Sample Numbers	**From: (Print name, Agency)**	**Release signature:**	**Release Date:**	**Delivered via:** ☐ **FedEx** ☐ **In Person** ☐ **Other:**
	To: (Print name, Agency)	**Receipt signature:**	**Receipt Date:**	
Sample Numbers	**From: (Print name, Agency)**	**Release signature:**	**Release Date:**	**Delivered via:** ☐ **FedEx** ☐ **In Person** ☐ **Other:**
	To: (Print name, Agency)	**Receipt signature:**	**Receipt Date:**	
Sample Numbers.	**From: (Print name, Agency)**	**Release signature:**	**Release Date:**	**Delivered via:** ☐ **FedEx** ☐ **In Person** ☐ **Other:**
	To: (Print name, Agency)	**Receipt signature:**	**Receipt Date:**	
Sample Numbers	**From: (Print name, Agency)**	**Release signature:**	**Release Date:**	**Delivered via:** ☐ **FedEx** ☐ **In Person** ☐ **Other:**
	To: (Print name, Agency)	**Receipt signature:**	**Receipt Date:**	
Sample Numbers	**From: (Print name, Agency)**	**Release signature:**	**Release Date:**	**Delivered via:** ☐ **FedEx** ☐ **In Person** ☐ **Other:**
	To: (Print name, Agency)	**Receipt signature:**	**Receipt Date:**	

USFWS WGL Sample Receipt Checklist

Case Number:	Receipt Date:
Other:	Rec'd by:

Were samples shipped? Yes, FEDEX/UPS/Other No, Courier pickup/hand delivered	Comments:
Cooler temp upon arrival _____^{0}C/NA	
Chain-of-custody (COC) present? Yes/no Complete? Yes/no	
Custody seals present on cooler? Yes/no Samples? Yes/no	
Were sample containers intact? Yes/no	
Samples and COC match? Yes/no	
If any problems, was project manager notified? Yes/no By whom?_____	
Appropriate sample containers? Yes/no	
Date/time of collection on COC Yes/no	
Location and ID of sample storage: Freezer_____ Refrigerator_____	
Temperature log updated for storage? Yes/no	

Screen capture of Excel log file. First worksheet in workbook outlines log for all sampling events or cases which correspond to samples from a particular system. Subsequent worksheets are created for each case and allow for sample tracking through the eDNA lab.



WGL eDNA Processing Case Log 2013.xlsx - Microsoft Excel

Processing Priority	Case Number	Number of Samples	System	Status (Open/Closed)
1	13-GL-1002	100	Great Lakes	open
2	13-GL-1001	300	Great Lakes	open
3	13-GL-1000	75	Great Lakes	open
4	13-GL-1003	200	Great Lakes	received
5	13-GL-1004	150	Great Lakes	received
6	13-GL-1005	200	Great Lakes	received
7	13-GL-1006	100	Great Lakes	
8	13-GL-1007	100	Great Lakes	
9	13-GL-1008	50	Great Lakes	
10	13-GL-2001	50	Great Lakes	
11	13-GL-2002	50	Great Lakes	
12	13-GL-2003	25	Great Lakes	
13	13-GL-2004	50	Great Lakes	
14	13-GL-2005	25	Great Lakes	
15	13-GL-2006	25	Great Lakes	
16	13-GL-2007	25	Great Lakes	
17	13-GL-2008	25	Great Lakes	
18	13-GL-2009	50	Great Lakes	
19	13-GL-2000	50	Great Lakes	
20	13-GL-3000	25	Great Lakes	
21	13-GL-3001	25	Great Lakes	
22	13-CAWS-1	60	CAWS	received
23	13-CAWS-2	60	CAWS	received
24	13-CAWS-3	60	CAWS	received
25	13-CAWS-4	60	CAWS	received
26				
27				
28				
29				
30				
31				
32				
33				
34				
35				
36				
37				
38				
39				
40				
41				
42				

Total Number of Samples 1940 — **Samples Completed** 0 / 1940

Total Number of Open Cases 3 — **Completed Cases** 0 / 3

Total Number of Received Cases 6

Case number assignment key

FY - Water Basin - Case Number - Sample Number

GL = Great Lakes
BR = Big Rivers
CAWS = Chicago Area Water System
sample numbers assigned as: -001, - 002, -003, etc.

Enter 3-month dilution series case number assignment key

FY - 3MDS - QTR1 (or QTR 2, QTR 3, QTR 4)

3MDS = 3 month dilution series
sample numbers assigned as: -001, - 002, -003, etc.

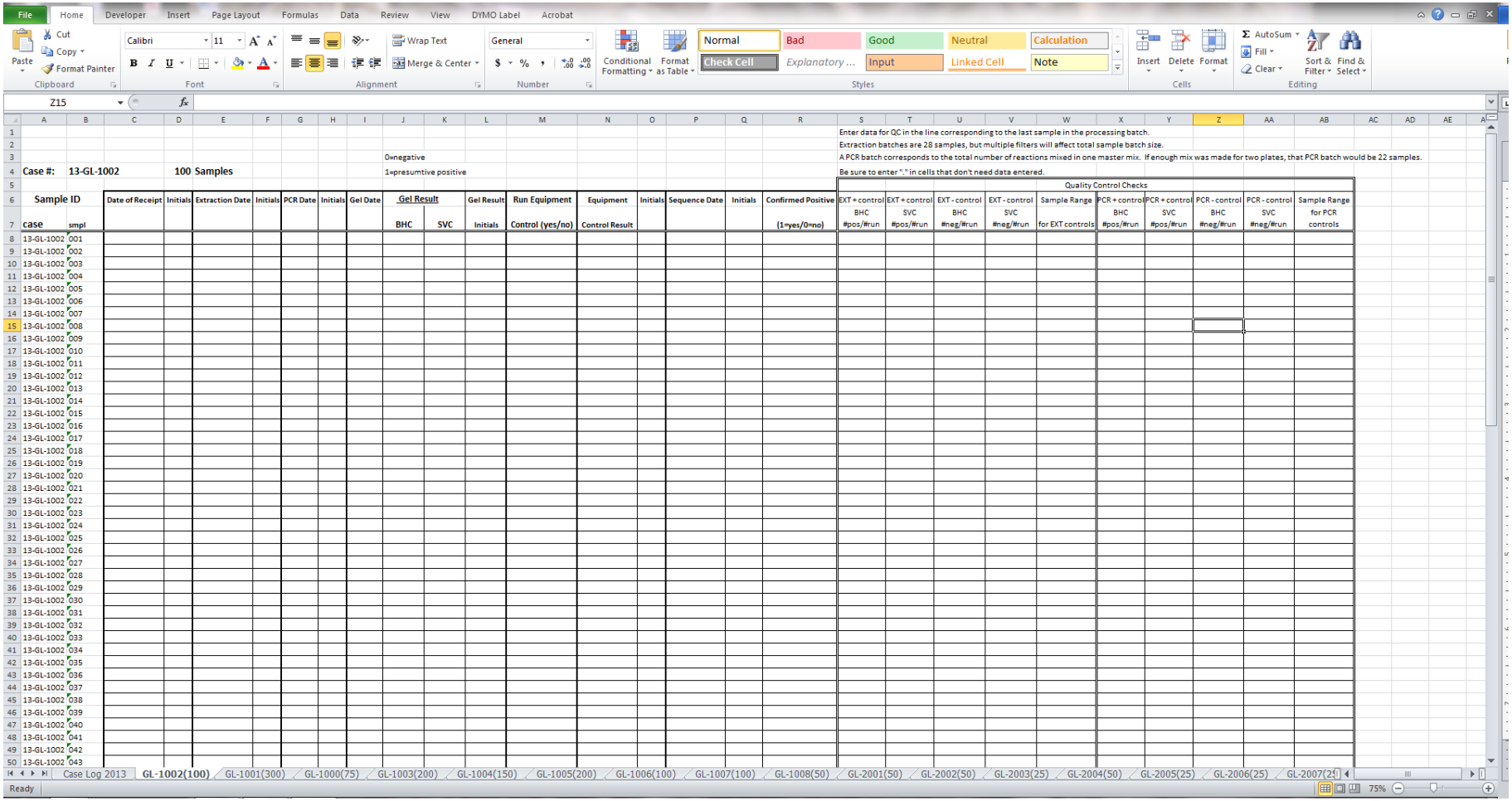
Unique ID for samples as follows:
eg. **13-GL-1002-003** = 2013 Great Lakes location #1002 sample #3
eg. **13-3MDS-QTR3-007** = 2013 3 month dilution series for April-June sample #7

Unique ID for equipment control samples as follows:
eg. **13-GL-1002-003-EC**

Case Log 2013 | GL-1002(100) | GL-1001(300) | GL-1000(75) | GL-1003(200) | GL-1004(150) | GL-1005(200) | GL-1006(100) | GL-1007(100) | GL-1008(50) | GL-2001(50) | GL-2002(50) | GL-2003(25) | GL-2004(50) | GL-2005(25) | GL-2006(25) | GL-2007(25)

Ready

Screen capture of Excel log file with the first case sample log open. Samples are tracked by date and process step through the lab. Corresponsing quality control results by extraction batch and PCR batch are reported with sample results.



Case #: 13-GL-1002 100 Samples

0=negative
1=presumtive positive

Enter data for QC in the line corresponding to the last sample in the processing batch.
Extraction batches are 28 samples, but multiple filters will affect total sample batch size.
A PCR batch corresponds to the total number of reactions mixed in one master mix. If enough mix was made for two plates, that PCR batch would be 22 samples.
Be sure to enter "." in cells that don't need data entered.

Sample ID		Date of Receipt	Initials	Extraction Date	Initials	PCR Date	Initials	Gel Date	Gel Result		Gel Result	Run Equipment	Equipment	Initials	Sequence Date	Initials	Confirmed Positive	Quality Control Checks									
case	smpl								BHC	SVC	Initials	Control (yes/no)	Control Result				(1=yes/0=no)	EXT + control BHC #pos/#run	EXT + control SVC #pos/#run	EXT - control BHC #neg/#run	EXT - control SVC #neg/#run	Sample Range for EXT controls	PCR + control BHC #pos/#run	PCR + control SVC #pos/#run	PCR - control BHC #neg/#run	PCR - control SVC #neg/#run	Sample Range for PCR controls
13-GL-1002	001																									
13-GL-1002	002																									
13-GL-1002	003																									
13-GL-1002	004																									
13-GL-1002	005																									
13-GL-1002	006																									
13-GL-1002	007																									
13-GL-1002	008																									
13-GL-1002	009																									
13-GL-1002	010																									
13-GL-1002	011																									
13-GL-1002	012																									
13-GL-1002	013																									
13-GL-1002	014																									
13-GL-1002	015																									
13-GL-1002	016																									
13-GL-1002	017																									
13-GL-1002	018																									
13-GL-1002	019																									
13-GL-1002	020																									
13-GL-1002	021																									
13-GL-1002	022																									
13-GL-1002	023																									
13-GL-1002	024																									
13-GL-1002	025																									
13-GL-1002	026																									
13-GL-1002	027																									
13-GL-1002	028																									
13-GL-1002	029																									
13-GL-1002	030																									
13-GL-1002	031																									
13-GL-1002	032																									
13-GL-1002	033																									
13-GL-1002	034																									
13-GL-1002	035																									
13-GL-1002	036																									
13-GL-1002	037																									
13-GL-1002	038																									
13-GL-1002	039																									
13-GL-1002	040																									
13-GL-1002	041																									
13-GL-1002	042																									
13-GL-1002	043																									

Example of -20 freezer temperature log.

Freezer Temperature Log

Month: _____ Year: _____

Freezer ID: _____ Freezer Location: _____

Day	Displayed Temp	Date	Initials	Maintenance (Y/N)
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				
26				
27				
28				
29				
30				
31				

Temperature should be between -23 and -17 °C for recording.

Daily log for cold storage coolers, used in processing trailers to keep samples cold until shipment to WGL.

Cooler Temperature Log

Case#s: _____

Sample IDs	Date	Time (am/pm)	Displayed Temp	Initials

Example of -80 freezer temperature and sample storage log. All cold storage appliances that store samples after receipt but before extraction, or store archived extracts have a temperature log and a sample log. The temperature log is filled out daily and the sample log each time samples are removed or stored.

Ultra Low Freezer Temperature Log

Month: _____ Year: _____

Freezer ID: _____ Freezer Location: _____

Day	Temp in range (Y/N)	Date	Initials	Maintenance (Y/N)
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				

-80 °C Sample Log

Freezer ID and Location: _____

Line Number	Case # and sample numbers CHECKED IN	Sample number range, state action taken (ex: extraction). Date and Initial CHECKED OUT.			

Example of ambient temperature (alcohol-preserved centrifuge samples only) sample storage log. The sample log is filled out each time samples are removed or stored.

Bench-top Shelving Log

Room _____

Line Number	Case # and sample numbers CHECKED IN	Sample number range, state action taken (ex: extraction). Date and Initial CHECKED OUT.			

Thermocycle log

PCR Log

Month: _____ Year: _____

Machine ID: _____ Machine Location: _____

Plate ID	PCR Machine ID	Plate Orientation	PCR Program	Run Start Time	Run Finish Time	Date/Initials

Lab data sheets for extraction, amplification, gel loading, and sequencing.

Extraction data sheet: to be filled out and taped into extraction room data book

Analyst_____ Date_____ Sample batch_____

Extraction room # 124 or 125 (circle)

Reagents used: note lot ID and expiration date for kit components

Reagent/tubes	**Lot #**	**Expiration date**
ATL		
Proteinase K		
AL		
Ethanol		
AW1		
AW2		
Molecular grade water		
2-mL collection tube		
Spin filter		

Start time_____

Tube ID	**Sample ID**	**Number Filters**		**Tube ID**	**Sample ID**	**Number Filters**
1				16		
2				17		
3				18		
4				19		
5				20		
6				21		
7				22		
8				23		
9				24		
10				25		
11				26		
12				27		
13				28		
14				29	Extraction Negative	1
15				30	Extraction Positive	1

Notes for any deviations from QAPP or lab blunders:

_____

_____

_____

_____

_____Finish time:_____

PCR data sheet: to be filled out and taped into PCR room data book

Analyst(s)_____ Date_____ Sample batch_____ Start time_____

Reagents and recipe: note batch (for diluted working primers), or lot ID and expiration date

Reagent name	Volume per rxn	Volume for ___ rxns	Lot or ID #	Expiration date
10X PCR buffer	2.5 µl	µl		
dNTP (10 mM mixed)	0.5 µl	µl		
MgCl (50 mM)	0.75 µl	µl		
Forward primer (10µM)	0.5 µl	µl		
Reverse primer (10µM)	0.5 µl	µl		
Platinum *taq* (5U/ µl)	0.25 µl	µl		
Sterile ddH_2O	19 µl	µl		

Positive control ID_____ Negative control _____

Silver (SV) or Bighead (BH) or Both (circle) use SV or BH or both in plate name

Plate Name_____ Date_____

Case ID#_Sample ID #-#_SPECIES_INITIALS_DDMONYY

	1	2	3	4	5	6	7	8	9	10	11	12
A												**P**
B												**P**
C												**P**
D												**P**
E												**E**
F												**N**
G												**N**
H												**N**

Notes for any lab blunders or deviations from QAPP:

_____
_____
_____
_____
_____
_____

Cycler ID _____ Did cycle complete Y N

Time started_____ Time removed_____

Exhibit 13 page 2

Plate Name_____ Date_____

Cycler ID _____ Did cycle complete Y N

Plate Name_____ Date_____

Case ID#_Sample ID #-#_SPECIES_INITIALS_DDMONYY

Case ID#_Sample ID #-#_SPECIES_INITIALS_DDMONYY

	1	2	3	4	5	6	7	8	9	10	11	12
A												**P**
B												**P**
C												**P**
D												**P**
E												**E**
F												**N**
G												**N**
H												**N**

	1	2	3	4	5	6	7	8	9	10	11	12
A												**P**
B												**P**
C												**P**
D												**P**
E												**E**
F												**N**
G												**N**
H												**N**

Cycler ID _____ Did cycle complete Y N

Plate Name_____ Date_____

Case ID#_Sample ID #-#_SPECIES_INITIALS_DDMONYY

	1	2	3	4	5	6	7	8	9	10	11	12
A												**P**
B												**P**
C												**P**
D												**P**
E												**E**
F												**N**
G												**N**
H												**N**

Cycler ID _____ Did cycle complete Y N
Time started_____ Time
removed_____

Gel data sheet: thermal print of gel should be taped on the facing page. Sign across the photo and this page. Exhibit 13

Analyst(s)_____ Date_____ Plate names: _____ _____

Lane	Gel #	+	Sample ID	+	Gel #	+	Sample ID	+

#	Sample ID				Sample ID			
M	ladder		ladder		ladder		ladder	
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								
18								
19								
20								
21								
22								
23								
24								
M	PCR +		PCR +		PCR +		PCR +	
M	ladder		ladder		ladder		ladder	
25								
26								
27								
28								
29								
30								
31								
32								
33								
34								
35								
36								
37								
38								
39								
40								
41								
42								
43								
44								
45								
46								
47								
48								
M	PCR +		PCR +		PCR +		PCR +	

Seq data sheet: to be filled out and taped into Seq room data book Exhibit 14

Analyst_____ date_____ Kit or sample batch_____

Reagents and recipe for separate forward and reverse reactions: note batch (for diluted working primers), or lot ID and expiration date

Reagent name	Volume per rxn	Volume for ___ rxns (f & r mixes)	Lot or ID #	Expiration date
5X sequencing buffer	4.0 µl	µl		
Forward OR reverse primer (10µM)	0.8 µl	µl		
BigDye V3.1	1.0 µl	µl		
ddH2O	10.2 µl	µl		

Indicate which species in sample ID

Plate Name_____ Date_____

Case#_ddMONyy_Initials

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Notes for any lab blunders or deviations from QAPP:

_____

_____

_____

_____

_____Cycler ID _____ Did cycle complete Y N

Time started_____ Time removed_____

Exhibit 15

Quality Assurance Project Plan Certification Statement

I, the undersigned, certify that I have read and that I understand the Quality Assurance Project Plan (QAPP) for the eDNA monitoring of Invasive Asian Carp. I further certify that I will follow the procedures listed in this QAPP.

Signed: _____

_____

Name Agency Date

Exhibit 16

Quality Control Audit Checklist for Field Sampling and Water Processing:

I, _____, observed sampling processing for samples
Sampling Quality Lead
_____collected on _____, from the

following locations:_____,

by the following personnel_____.

During this time, I compared the actions of the crew to the QAPP and witnessed adherence to the QAPP in the following: A check indicates compliance with the QAPP. An X indicates the QAPP was not followed and a short explanation follows. If more space is needed, attach a written report.

Contamination Prevention:

Boat and equipment preparation _____

Sample collection and navigation_____

I, _____, observed sampling processing for samples
Processing Quality Lead
_____Collected on _____, from the

following locations:_____,

by the following personnel_____.

During this time, I compared the actions of the crew to the QAPP and witnessed adherence to the QAPP in the following: A check indicates compliance with the QAPP. An X indicates the QAPP was not followed and a short explanation follows. If more space is needed, attach a written report.

Contamination Prevention:

Sample processing equipment_____

Sample processing procedures_____

Explanation for deviation(s) from the QAPP:_____

_____

_____

_____

_____

Appendix A

Roles and Responsibilities

And

Annual Staff Assignments

As of April 30, 2014, the following staff are assigned to the eDNA monitoring project.

eDNA Program Coordinator: Kelly Baerwaldt, USFWS

eDNA Processing Leader: Emy Monroe, WGL, 608-783-8402

DNA Processing Quality Assurance Specialist: Maren Tuttle-Lau, WGL, 608-783-8403

Data Documentation & Reporting Specialist: Jennifer Bailey, WGL, 608-783-8451

Fish and Wildlife Conservation Office Points of Contact:

La Crosse: Ann Runstrom, 608-783-8433

Columbia: Patty Herman, 573-234-2132 x 170

Carterville: Sam Finney, 618-997-6869 x 17

Green Bay: Mark Holey, 920-866-1760

Ashland: Mark Brouder, 715-682-6185 x11

Alpena: Scott Koproski, 989-356-3052 x 1023

USFWS Midwest Region
eDNA Roles and Responsibilities
April 2014

ROLES

Regional Office

Region 3 Deputy RD/National Asian Carp Lead: Charlie Wooley
Fisheries ARD: Todd Turner
Deputy ARD/National Asian Carp Plan Coordinator: Aaron Woldt
R3 AIS Coordinator: Mike Hoff
R5 AIS Coordinator: Sandra Keppner
R3 Asian Carp/eDNA Coordinator: Kelly Baerwaldt
R3 FWCO Program Supervisor: Maureen Gallagher

Whitney Genetics Lab

Supervisory Molecular Geneticist: Vacant
Molecular Geneticist: Emy Monroe

FWCO's

Project Leaders: Mark Holey, Mark Brouder, Scott Koproski, Rob Simmonds, Scott Yess (Acting La Crosse), Kofi Fynn-Aikins

eDNA POC's: Each FWCO has identified an eDNA Point of Contact from each office. These POC's are—Alpena: Chris Olds; Green Bay: Tim Strakosh; Ashland: Mark Brouder (or designee); Carterville: Jeff Stewart; Columbia: Patty Herman; LaCrosse: Nick Bloomfield; Lower Great Lakes (Region 5): Sandy Keppner

****

RESPONSIBILITIES

Deputy ARD/National Asian Carp Plan Coordinator:
Regional oversight of all Asian carp and eDNA activities. Senior level authority on all decisions on eDNA sampling program.

Region 3 Aquatic Invasive Species Coordinator:
National Aquatic Invasive Species Subject Matter Expert on development and implementation of processes and strategies for aquatic invasive species (AIS) management, providing regional project leadership on DOI initiatives addressing AIS management. Provides technical assistance for development and application of new scientifically based concepts and improved technology in AIS prevention and control. Leads interagency and interregional efforts to develop and implement programs for early detection and rapid response processes.

Region 3 Asian Carp/eDNA Coordinator:
Regional Office staff specialist responsible for development, coordination and implementation of the Service's eDNA monitoring program for Asian carps. Assumes principal responsibilities for initiating, leading, facilitating, integrating, coordinating, and communicating necessary monitoring work and activities using eDNA of the Midwest Region's Fisheries Program through the cooperative conservation community. Provides guidance for implementing and utilizing eDNA activities at the field level. Provides technical leadership to plan, conduct and lead other biologists as well as coordinate activities to identify and coordinate surveillance areas in water bodies for the early detection of Asian carp eDNA. Ensures the best scientific practices are used during the development and implementation of management plans to monitor, control, and eradicate Asian carp. Implements and coordinates the eDNA collection program; eDNA processing and interpretation of data; collection and dissemination of research information from institutions and research agencies on findings and new developments in eDNA collection and interpretation of data and the development and implementation of Asian carp management plans. Responsible for the overall QAPP and its

implementation at the field and lab levels. Responsible for communication between the lab and Regional office. Integration with interagency ECALS team and other relevant entities (Academia, etc). Responsible for external communication with partners and updates on sampling and lab (results) status.

Region 3 FWCO Program Supervisor:
This position exercises supervisory line authority in management of the Region 3 FWCO's. In addition to managing the assigned field stations, serves the role of coordinating and integrating their operations with other Fishery Program operations, and ensuring cooperative and productive partnerships with the Upper Midwest and Great Lakes Landscape Conservation Cooperative and between the Midwest and Northeast Regions.

Supervisory Molecular Geneticist, Whitney Genetics Lab:
Responsible for planning and implementing operations of a complex Genetics Laboratory providing lab services to federal and state entities nationwide. Supervises a staff of fish biologists and technicians performing a variety of procedures in support of early detection and monitoring of aquatic species and in meeting fisheries management objectives. Responsible for all processing of eDNA samples and reporting of results to eDNA coordinator and supervisor. Primary author and editor of all processing related portions of QAPP.

Project Leaders:
These positions exercise line and management responsibility for their respective office to protect, enhance, and conserve fish and wildlife resources through a variety of activities. Among other duties, incumbent carries out all aspects of administration of the Aquatic Nuisance Species Act in the states or geographic area of responsibility assigned to the respective office. Incumbent will report any issues/ questions to eDNA coordinator and/or National AC coordinator.

eDNA POC's
Serve as lead office representative for eDNA activities at their respective station. Also serve as representatives of their respective stations on the eTeam. eDNA POC from each FWCO is also that office's coordinator with partners and responsible for developing and implementing station sampling plans, to be provided to eDNA coordinator and Regional Office.

****

STRUCTURE AND FUNCTION OF THE eTEAM (eDNA Team)

Purpose: Internal planning and review of field implementation of eDNA sampling in the region. Facilitate communication between FWCO field staff responsible for eDNA sampling in a structured forum. Facilitate communication with RO staff responsible for Asian carp surveillance activities.

Members:

Whitney Genetics Lab Leader (Emy Monroe)

eDNA Coordinator (Kelly Baerwaldt)

FWCO eDNA POC's (Alpena: Chris Olds; Green Bay: Tim Strakosh; Ashland: Mark Brouder; Carterville: Jeff Stewart; Columbia: Patty Herman; LaCrosse: Nick Bloomfield; Lower Great Lakes: Sandy Keppner)

Asian Carp Coordinator (Aaron Woldt)

Regional FWCO Program Supervisor (Maureen Gallagher)

Goal of eTeam: Facilitate communication regarding the field implementation and execution of the QAPP for eDNA between the FWCO eDNA points of contact (including R5) and with the RO.

Responsible for reviewing and recommending changes to the field sampling portions of the QAPP for eDNA. Responsible for keeping respective Project Leaders apprised of team progress. Responsible for developing site specific sampling plans in coordination with state partners. Responsible for developing, standardizing, and executing training of field staff. Will meet regularly or as needed to discuss field issues, resolutions, and lessons learned regarding field sampling of eDNA.

eTeam will be chaired by the eDNA Coordinator (Kelly Baerwaldt), and all actions and recommendations from the group must be approved by the Asian Carp Coordinator (Aaron Woldt). Emy Monroe from Whitney Genetics Lab will serve as the lead from the Lab. Chris Olds (Alpena POC) will serve as the lead from the FWCO's. The role of field lead of the eTEAM may be rotated among field stations per the discretion of the eTeam members.

Appendix B

Internal Communication of Results SOP

Standard Operating Procedure:
Notification of U.S. Fish and Wildlife Service eDNA Results Communication Plan

The 2014 communication plan is currently being updated based on comments from the States and the Council of Great Lakes Fishery Agencies. The updated plan will be distributed before eDNA samples for 2014 are processed and results made available to agencies and the public.

This plan will be made available on: http://www.fws.gov/midwest/fisheries/eDNA.html

Appendix C

Data Management

1. Purpose

In order to keep accurate records of eDNA sample collections, personnel associated with sampling and processing of collections, and data associated with a specific collection sample, datasheets associated with sample collections must be kept in accordance with the following protocols for quick reference and to prevent loss. This appendix describes procedures for data reporting and data management specific to USFWS.

2. Data management

(1) Field data and data sheets are the responsibility of the FWCO.

a. FWCO staff are responsible for entering field data, proofing data and maintaining data at their location. Hard copy and electronic data should be backed up regularly.
b. Field data sheets should be scanned and saved in a PDF with all of the data sheets for a particular location/case in one document. The filename should include sampling dates and location information, and should be saved on the station's shared drive as well as attached to the database described in #3.
c. Original COC forms are sent to WGL with the samples. FWCOs will receive a scanned COC that should be printed and filed in a project binder and the electronic copy saved with electronic field data and scanned data sheets.
    i. The original COC forms are kept in a project binder at WGL, and will be given to the eDNA Project Coordinator at the end of each sampling year.

(2) Lab data and data sheets are the responsibility of WGL.

a. WGL will maintain hard copies of laboratory notebooks and the electronic project data file cataloging each case in an excel file. Gel photos are printed and placed into lab notebooks, and electronic files of the images are also saved on the lab network, which is backed up. Lab notebooks will be stored in a fireproof file cabinet. Sequence data will be saved electronically on the station's server.
b. WGL will scan data notebooks into PDFs by cases and these will be saved on the lab networked computer with a back-up as well as on the station's shared drive which is also backed up by IT staff.
c. Eventually an electronic Laboratory Information Management System will be utilized to track cases and samples through the lab workflow. The LIMS will be backed up a minimum of 4 times on separate hard drives.
    i. Final reports will be generated as each case is completed, sent to the eDNA Program Coordinator as well as saved electronically in the lab network and on the station's shared drive.
    ii. The PDFs of the scanned field COC as well as the gel images and sequencing results will be maintained in electronic copy on site with back-up.

(3) Collated field and lab data in geo-referenced database managed at the Regional Office

a. FWCOs are responsible for entering and proofing field data into the database within TWO weeks of collection.
b. WGL is responsible for filing final case reports of results with the eDNA Program Coordinator within 24 hours of result confirmation.
c. USFWS Midwest Region will maintain a database complete with field and lab data as well as electronic copies of reports provided to partner agencies and the public.

Fisheries Sampling Site Data Management Plan

Developed by Gabe DeAlessio and R3 Fisheries Program

April 11, 2014

Purpose: The Fisheries Sampling data is collected by multiple field stations and needs to be reported and consolidated into a single location for archiving and centralizing mapping needs. Using ArcGIS Server (SDE) will create a simplified workflow and repository for the data.

User Descriptions:

Fisheries Field Users – these users are responsible for collecting the samples, quality control of the data, and inputting the sampling data into the system. They will have editing permission for the eDNA_SAMPLE table and read permission to the eDNA_SAMPLE_RESULTS feature class. This designation will include one employee from each FWCO as listed below:
Alpena FWCO – Chris Olds
Ashland FWCO – Mark Brouder
Carterville FWCO – Jeff Stewart
Columbia FWCO – Patty Herman
LaCrosse FWCO – Nick Bloomfield
Green Bay FWCO – Tim Strakosh

Fisheries Data Steward – This person (and their backup) will work with RO GIS Staff (see below) and be responsible for updating the eDNA_RESULTS data and joining the eDNA_RESULTS table to the eDNA_SAMPLE_RESULTS feature class. This person and their backup will have editing permission for the RESULTS table, the eDNA_SAMPLE feature class, and the eDNA_SAMPLE_RESULTS feature class. Also, the Data Steward will be the primary contact for field stations on assistance with training, troubleshooting, data review and management as needed, and day to day operations.

Primary – Brian Elkington/Kelly Baerwaldt

Backup – Karla Bartelt

eDNA Coordinator - This person will work with the Whitney Genetics Lab (WGL) to receive results and transmit them to the Fisheries Data Steward. The eDNA Coordinator will also assist in Coordinating FWCO eDNA sampling for the Region. This person will not have access to the SDE initially but may be added as a Primary Fisheries Data Steward in the future dependent on work flow and overall efficiency of the system.

Primary – Kelly Baerwaldt

RO GIS – Responsible for initial data loading, managing user permissions and server side issues. The RO GIS staff will advise the Fisheries Data Steward on database creation and editing (including design, implementation and training) but is not responsible for day to day updating of the database. Once in

production mode, the RO staff will assist Field Users only if the Fisheries Data Steward is unable to resolve the issue.

Primary – Gabe DeAlessio

Workflow Overview:
Sample data is collected by field stations. The data are collected and recorded as outlined in the Quality Assurance Project Plan (QAPP), with the addition of the Regional Unique Identifier. While the physical samples are sent to the Whitney Genetics Lab (WGL) for analysis, the Fisheries Field Users are responsible for digitizing their raw data, ensuring data accuracy, and loading that data into the eDNA_SAMPLE feature class in the ArcGIS Server (SDE) using an excel file template provided by the Fisheries Data Steward. All data will be error checked prior to loading (See data upload instructions below) and will be loaded no more than 2 weeks post sampling event by the Fisheries Field Users to ensure it is entered prior to when results arrive at the RO from the WGL.
Upon receiving the results data from the WGL, via the eDNA Coordinator, the Fisheries Data Steward (or their backup) will load the results data into the eDNA_RESULTS table. Once the data is loaded, the Fisheries Data Steward will produce the necessary maps to be used to inform the affected partner. Concurrent with website posting, the Fisheries Data Steward will join the eDNA_RESULTS table with the eDNA_SAMPLE feature class. This join will add the results and sampling data to the eDNA_SAMPLE_RESULTS feature class (read only for Fisheries Field Users) and delete the results and sampling data from the eDNA_RESULTS table and eDNA_SAMPLE feature class. The eDNA_SAMPLE_RESULTS feature class will be considered the long term data archive for future reference as needed.

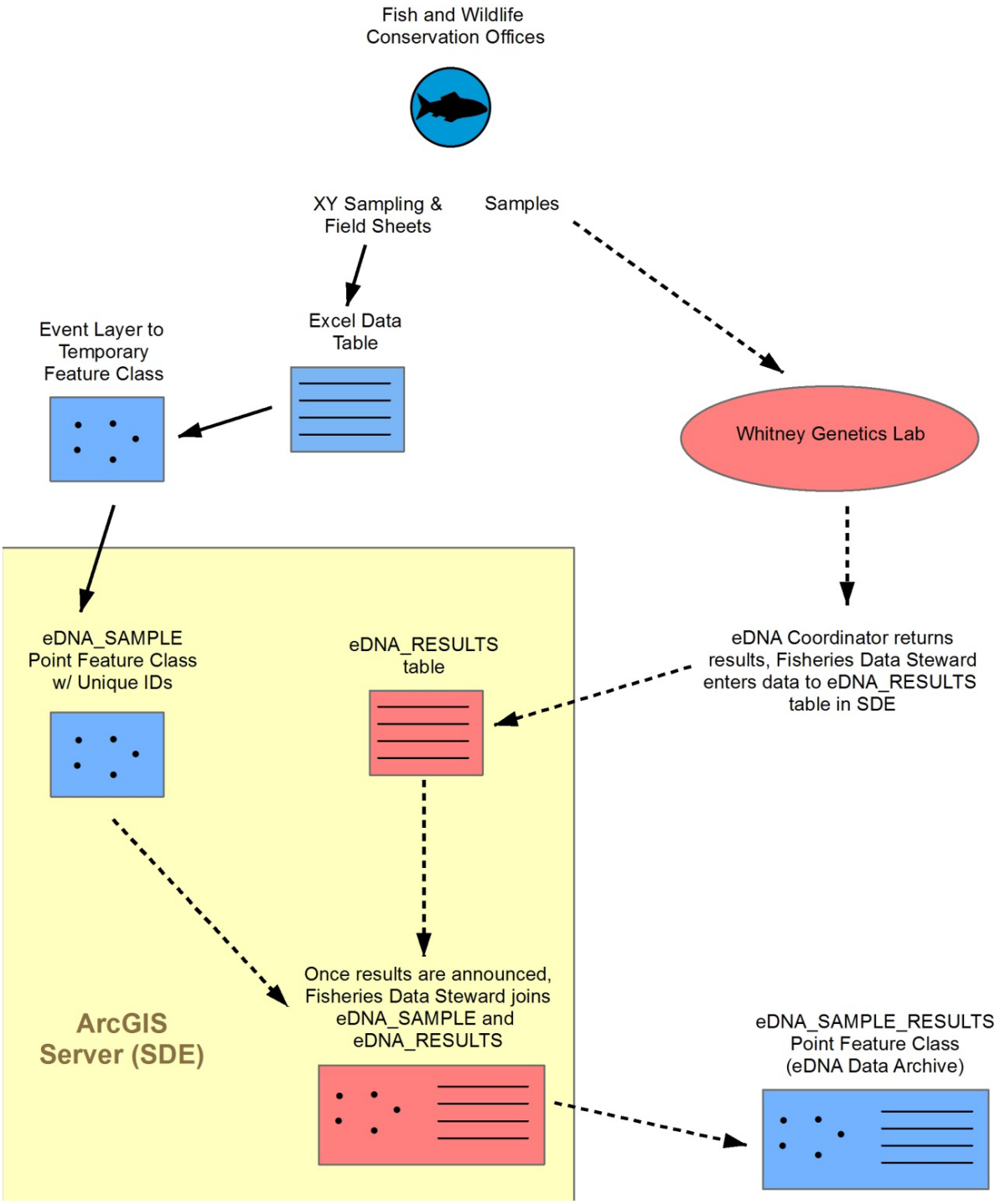


Figure 1. Workflow of eDNA sampling and results data into the Regional ArcGIS Server (SDE). The sampling data is entered by FWCO staff into the eDNA_SAMPLE point feature class, which contains all appropriate and standardized data fields (guidance provided in the Quality Assurance Project Plan (QAPP)). Corresponding results data is entered into a separate eDNA_RESULTS table once received from FHC and ultimately joined with the sampling data to create the eDNA_SAMPLE_RESULTS point feature class, a long term Regional eDNA data archive.

Table 1. Field descriptions for the eDNA_Sample point feature class.

Field Name	Alias	Type	Length	Description
RUID	Regional Unique ID	Long Integer	n/a (8)	Composite number that has a 5-digit "case" number (a series of unique numbers assigned by the WGL to each FWCO at the start of the sampling year and provided for each individual planned sampling event followed by a 3 digit sample ID number assigned by the FWCO numerically as they take samples in the field (no punctuation, #######)
FWCO_ID	Sampling Station ID code	Text	3	Alpena FWCO – ALP Ashland FWCO – ASH Carterville FWCO – CAR Columbia FWCO – COL Green Bay FWCO – GRB LaCrosse FWCO - LAX
STATE	State	Text	2	Two letter state code in which samples were taken.
BASIN	Basin	Text	4	LH = Lake Huron LM = Lake Michigan LE = Lake Erie LS = Lake Superior LO = Lake Ontario CAWS = Chicago Area Waterway System UMR=Upper Miss River OHR=Ohio river -Other designations to be added as needed by Fisheries Data Steward
WATERBODY	Waterbody	Text	100	Waterbody name
DATE_COLL	Collection Date	Date		Date of collection (MMDDYYYY)
WIND_DIR	Wind Direction	Text	20	Abbreviated wind direction, "Calm", "Variable", or shorthand (NE, NW, SE, SW, N, S, E, W) and wind speed if available.
LATITUDE		Double	n/a	WGS 84 Decimal Degrees, 5 decimals

LONGITUDE		Double	n/a	WGS 84 Decimal Degrees, 5 decimals
TEMP_F		Double	n/a	Temperature in degrees Fahrenheit (1 decimal)
DEPTH		Double	n/a	Depth where sample was taken in Feet (1 decimal)
DOUBLE_SAMPLE		Text	5	Yes/No
BLANK		Text	5	Yes/No
HABITAT		Text	30	Habitat type where sample was taken, i.e. LDB, RDB, MC, Bay, Lake, Confluence, etc.
COLLECT_TIME	Collection Time	Long Integer	4	Time stamp in military time, no punctuation
FILTER_TIME	Filter Time	Long Integer	4	Time stamp in military time, no punctuation
PROCESSOR		Text	3	Initials of person who filtered the sample
COMMENTS		Text	255	General notes, issues, or observations while sampling

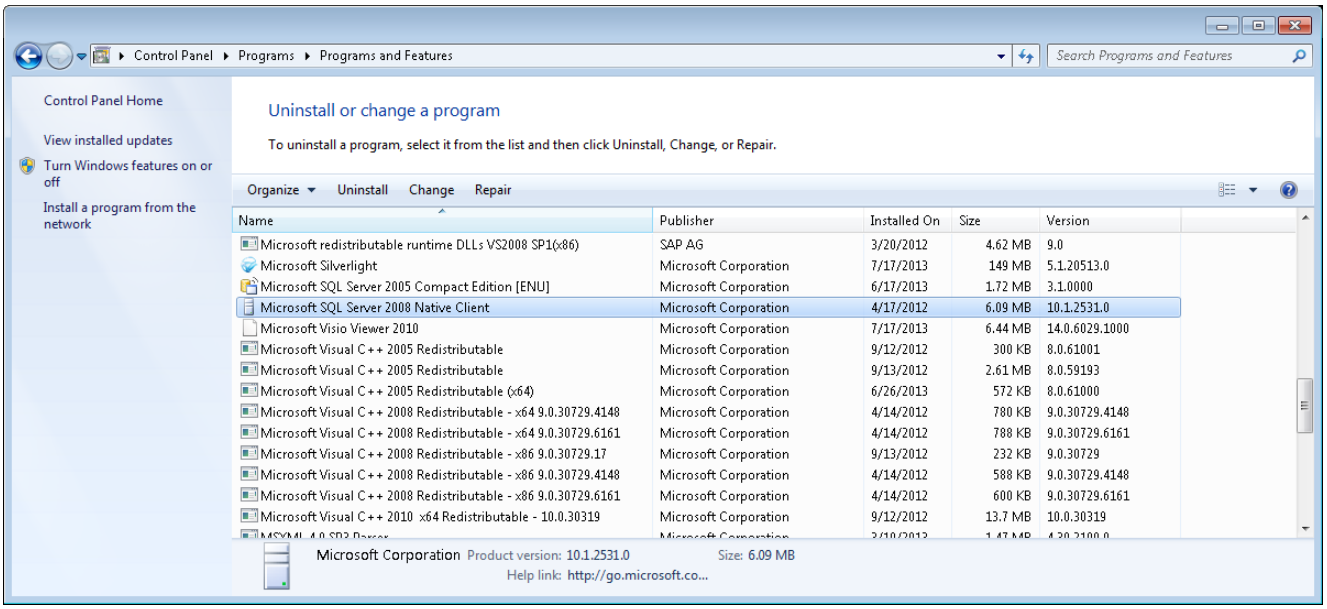
Table 2. Field descriptions for eDNA_Results table.

Field Name	Alias	Type	Length	Description
RUID	Regional Unique ID	Long Integer	n/a (8)	Composite number that has a 5-digit "case" number (a series of unique numbers assigned by the WGL to each FWCO at the start of the sampling year and provided for each individual planned sampling event followed by a 3 digit sample ID number assigned by the FWCO numerically as they take samples in the field (no punctuation, ########)
BIGHEAD		Text	5	Yes/No
SILVER		Text	5	Yes/No

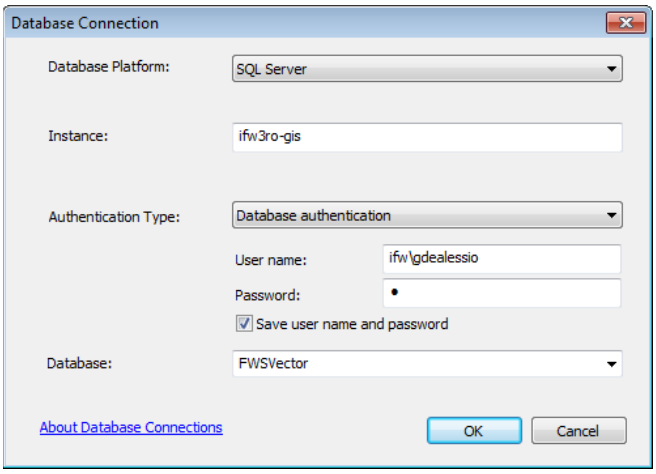
System Requirements:

ArcGIS 10.1, SP1. This is available from "FWS Apps to Go" and doesn't require elevated privileges to install. Contact IT with any problems.

SQL Server 2008 Native Client – This allows ArcGIS to connect to ArcGIS Server (SQL Server). Look in Program Files to see if it's installed (screenshot below). If not, contact IT (x5115) to get it installed prior to connecting.



Establishing a Connection to the USFWS Midwest Region ArcGIS Server:
Go to ArcCatalog. Scroll down in the Table of Contents to Database Connections
Click Add Database Connection.
Set up as following, replacing the User Name with your Active Directory Short Name. (Generally IFW\first initial last name)
Set the Password to “a” (it cannot be left blank). Note that the software is actually passing your Windows Login password, the “a” is just a placeholder.



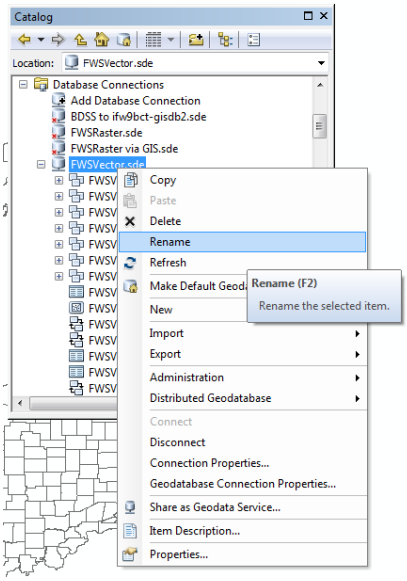
Click OK. You should be able to open the connection and see the various datasets.

Troubleshooting - if the connection fails, it is likely one of the following:

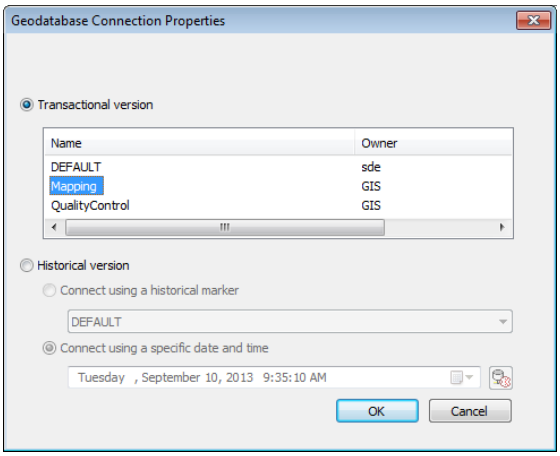
1) Double check your shortname and be sure all the parameters are correct.
2) You have not been granted permission to the database yet. Contact the RO GIS Staff (Gabe).
3) The SQL Server 2008 Native Client is not installed (see System Requirements).

Once the database connection is made, you should be able to see the many datasets.

Right click on the connection and choose RENAME. Change the name to FWSVector.



Lastly, we want to connect to the working version of the database. Right Click on the newly named FWSVector and choose Geodatabase Connection Properties.



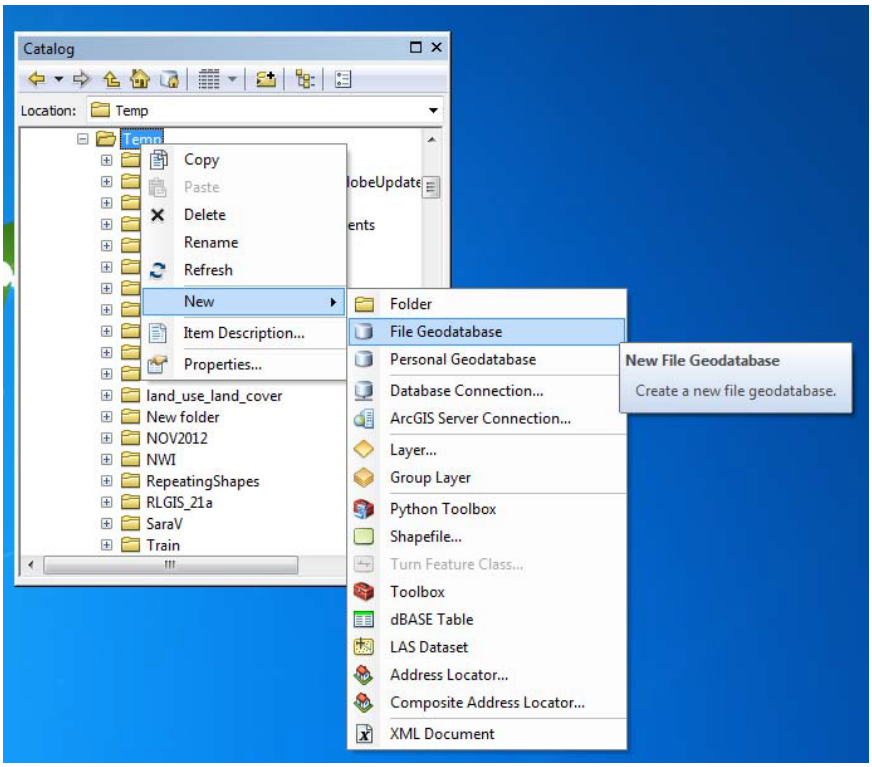
Select the Transactional version to Mapping and click OK.

Field User Instructions:

This section will guide you through the initial ArcMap set up and data loading process. Some of these steps can be done once as long as you save and reuse the ArcMap project for future data loads.

1) Create an empty geodatabase to store temporary data.

In ArcCatalog, select a location (suggested location > C:\TEMP) and right click and choose New > File Geodatabase



Rename the geodatabase: **Sample_Temp_Data.gdb**

2) Be sure your data is loaded into the field data MS Excel eDNA_SAMPLE datasheet.

Draft Master Table.xlsx [Read-Only]

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	
1	RUID	FWCO_ID	River	Date	Collector	Wind_Direction	Waypoint	Latitude	Longitude	Temp	Depth	Double	Blank	Habitat	Collect_Time	Filter_Time	Signature	Commen
2	GRB20130255	1004-001	Muskegon	5/13/2013	Strakosh	Variable	255	43.42337	-85.67512	53.7	4.6			RVR	1134	1310	CMO	
3	GRB20130256	1004-002	Muskegon	5/13/2013	Strakosh	Variable	256	43.42422	-85.67521	53.7	4.6			RVR	1136	1312	SG	
4	GRB20130257	1004-003	Muskegon	5/13/2013	Strakosh	Variable	257	43.42522	-85.67530	53.7	2			RVR	1138	1315		
5	GRB20130258	1004-004	Muskegon	5/13/2013	Strakosh	Variable	258	43.42565	-85.67551	53.7	2.7			RVR	1139	1317		
6	GRB20130259	1004-005	Muskegon	5/13/2013	Strakosh	Variable	259	43.42598	-85.67561	53.7	3.9			RVR	1140	1328	CMO	
7	GRB20130260	1004-006	Muskegon	5/13/2013	Strakosh	Variable	260	43.42622	-85.67566	53.5	1.5			RVR	1141	1332	CMO	
8	GRB20130261	1004-007	Muskegon	5/13/2013	Strakosh	Variable	261	43.42652	-85.67570	53.5	2.2			RVR	1142	1336	SG	
9	GRB20130262	1004-008	Muskegon	5/13/2013	Strakosh	Variable	262	43.42686	-85.67577	53.5	4.9			RVR	1143	1345		

eDNA_SAMPLE

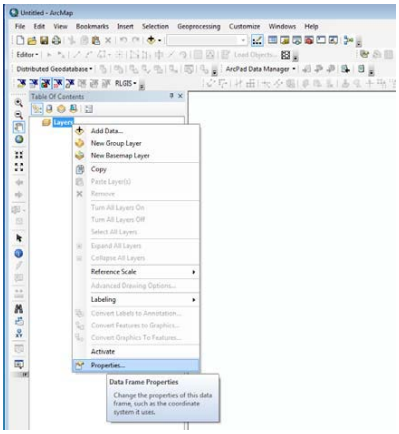
3) Open new ArcMap project.

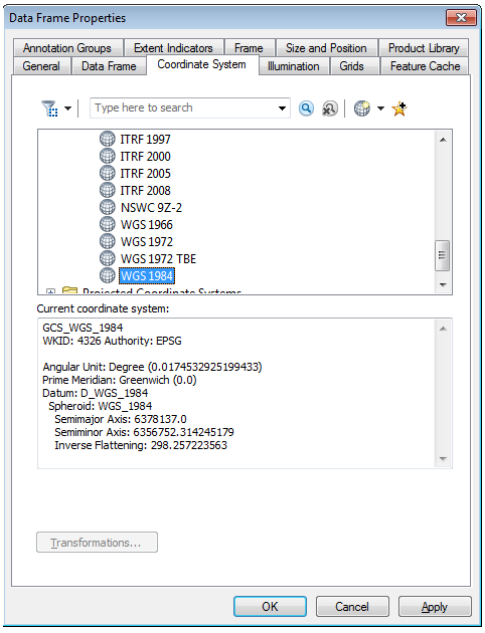
4) Right click on Layers > Data Frame Properties.

5) Choose the Coordinate System tab

6) Select Geographic Coordinate Systems >World > WGS 1984.

7) Click OK. This sets your ArcMap project to the coordinate system your XY data is in (Decimal Degrees - Lat/Long).

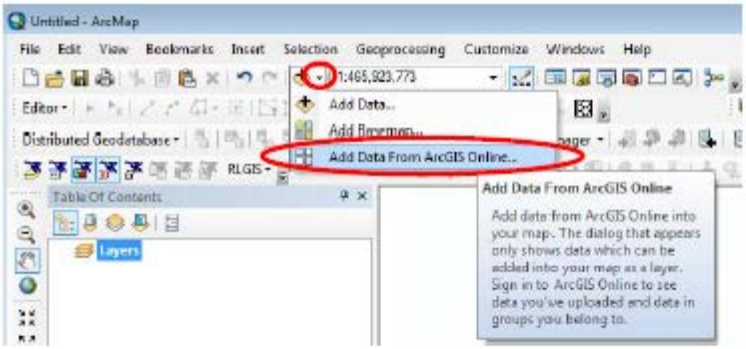




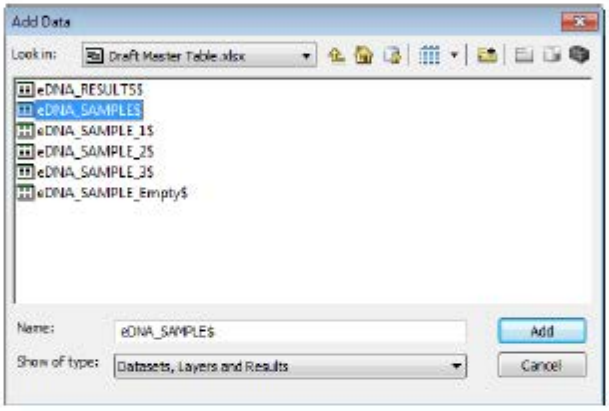
8) Next, add a basemap to your work area. If you have a local copy of a topographic or aerial, browse to that and add.

If not, you can use the Add Data Dropdown to choose Add Data from ArcGIS Online and type

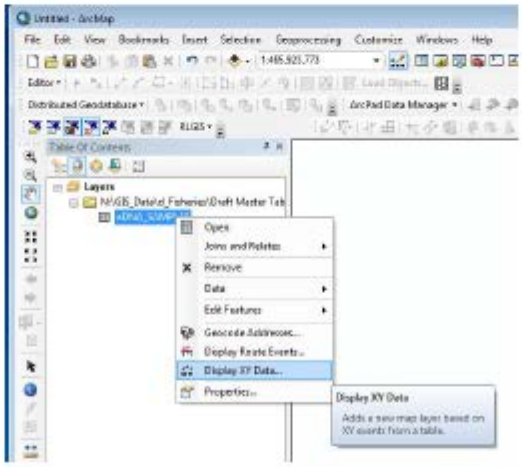
“Image” to locate a worldwide image set. (Note that slow internet speeds may be very slow to work with ‘live’ data.)



9) Use **Add Data** to bring the **eDNA_SAMPLE** datasheet into the Table of Contents.

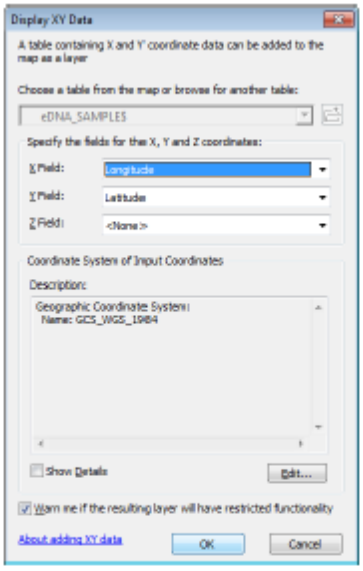


10) **Right Click** on the table and choose, **Display XY Data**.

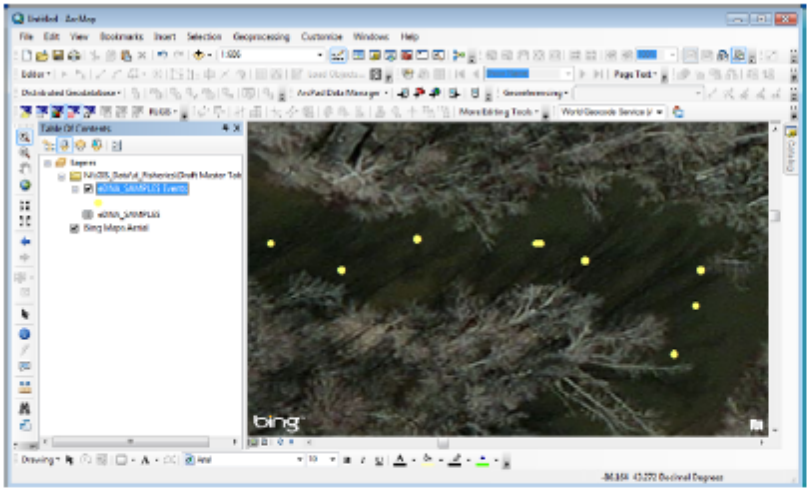


11) Latitude and Longitude will autopopulate. **Click OK**.

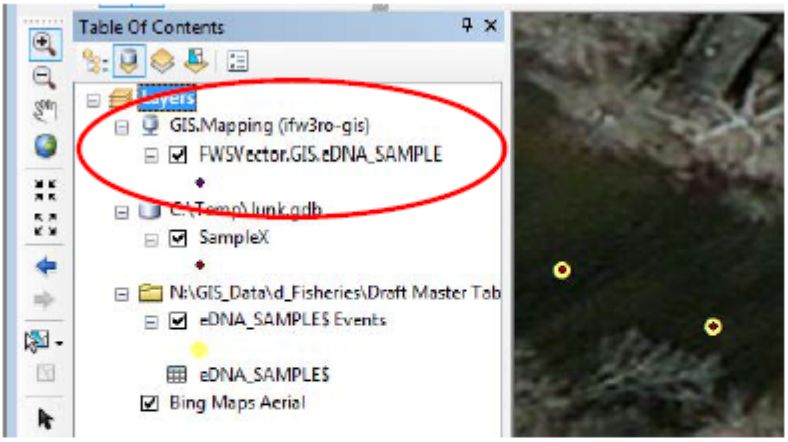
You will get a warning message regarding an Object ID field, **Click OK**.



12) Field users should Quality Check the data now. Review both positional accuracy as well as verifying all the required attributes are present and correct.



13) Once field QC is complete, right click on the Events layer and choose **Data > Export Data**

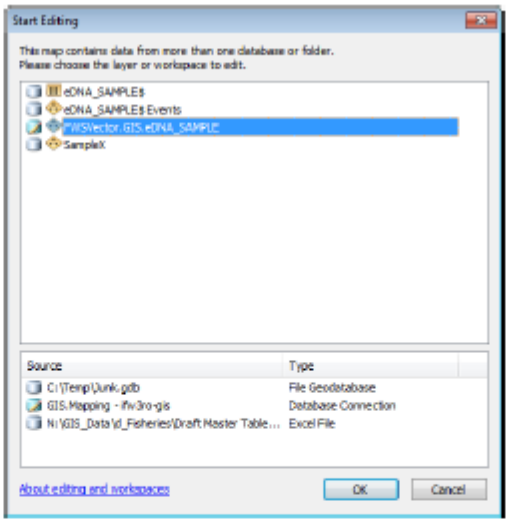


17) Make sure the Editor Toolbar is open. If it is not, go to **Customize>Toolbars>Editor**

Dock the toolbar where you prefer.

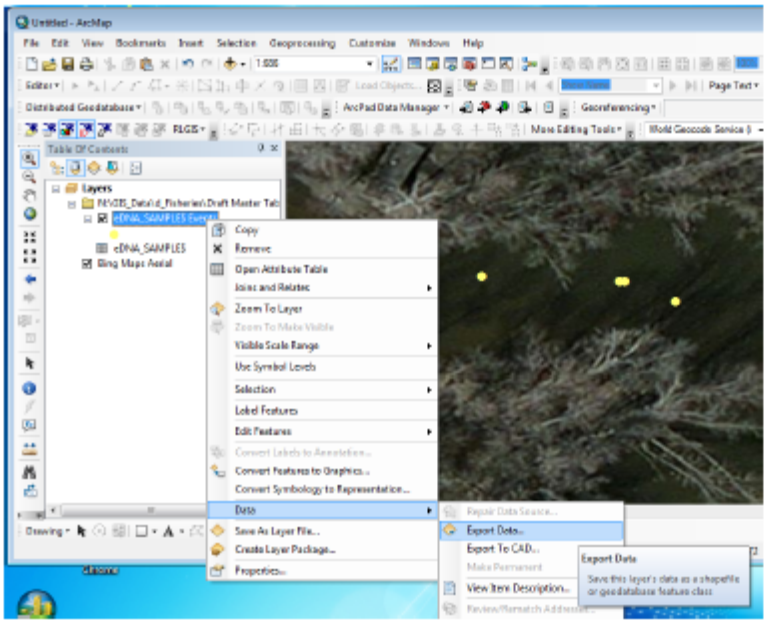
18) Click **Editor** > **Start Editing**.

Choose the FWSVector.GIS.eDNA_SAMPLE layer.



Click OK.

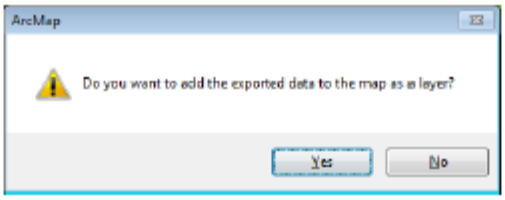
19) **Right click** on the SampleX layer. Choose **Selection** > **Select All**.



14) Browse to your Sample_Temp_Data.gdb and name the data SampleX

Click OK.

When asked if you want to add the data to the map, click Yes.

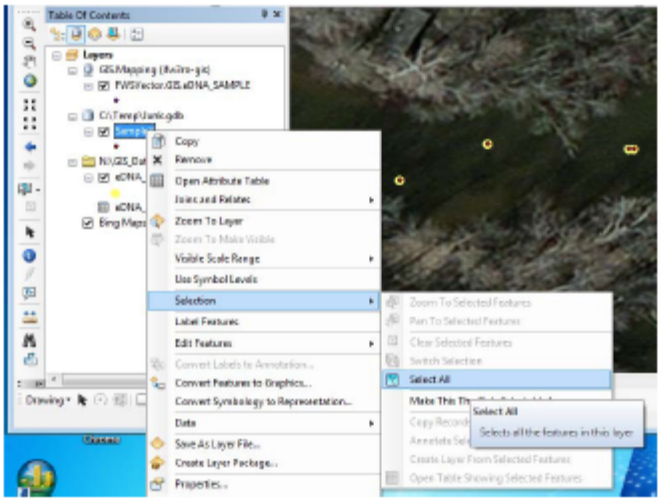


15) Add the eDNA_SAMPLE point feature class from the SDE server.

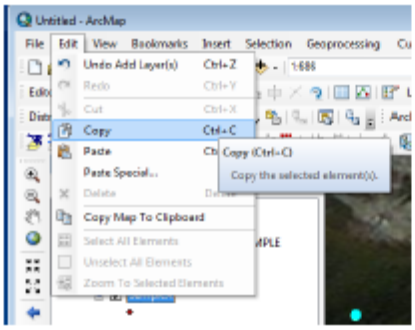
Click **Add Data**, then browse to **Database Connections > FWSVector.sde>eDNA_SAMPLE**

Click **Add**

16) Confirm the table of contents looks like the following, showing the GIS.Mapping version of the eDNA_SAMPLE point feature class.



20) On the Main Menu, choose **Edit** > **Copy**. (or Ctrl + C)



21) Next choose **Edit** > **Paste**. (or Ctrl + V)

Be sure your target dataset is the **eDNA_SAMPLE** point feature class.

Click OK.

22) **Turn off** the SampleX and Event point data in table of content.

Zoom to the newly added data, and review that the attributes pasted correctly.

23) On the Editor Toolbar, Save edits and Stop Editing.

24) You can right click and Remove the SampleX, Event data and the Excel eDNA_SAMPLE table. Your project is ready for the next data loading.

25) Go to File > Save to save the ArcMap project for reuse later. Note that you will now start on step 9 and skip steps 15 – 17 in future data loads if you use this .mxd.

Fisheries Data Steward Instructions (add more once names/fields are finalized):

Upon receiving lab results, copy/paste records into RESULTS table in ArcGIS Server/FWSVector

Appendix D

Sample Bottle Management Plan

The WGL study testing used sampling containers (report XXXX on file with eDNA Program Coordinator) demonstrated that sample bottles can be reused for eDNA sampling, but recommendations were for implementation of a decontamination specialist to oversee and confirm that decontamination was conducted according to the QAPP. The Regional Office has decided that WGL will serve as the single decontamination facility for the Asian carp eDNA monitoring program.

WGL will clean and prepare bottles according to the QAPP and store them in the lab in a clean room. WGL will package bottles according to the following procedure, and send them as needed to the field offices.

Empty bottles for field samples:

(1) New card board boxes and plastic liners will be purchased by WGL. Boxes will be assembled as needed and lined with the plastic bags.
(2) Cleaned and autoclaved bottles will be securely closed and placed in the bag.
(3) Bags will be sealed with tamper evident tape, boxes sealed and shipped to field stations. Water-filled cooler blanks may need to be sent separately, but all efforts will be made to get cooler blanks distributed at the annual training meeting each spring (if it is held in La Crosse).
(4) Field stations will receive boxes, store sealed until they are ready for labeling.
(5) Field stations will prepare coolers according to the QAPP, in as clean of an area as possible at the station.
    a. Anytime boxes are opened, staff should be in clean clothes and use a new pair of clean gloves for actually handling the bottles. For example, you may open the box with un-gloved hands. Then, put on gloves to open the bag and touch the bottles.
(6) Once clean coolers are ready, boxes may be opened, bottles labeled, and placed into clean coolers.
(7) After use, field staff can use the same bag and box to return the bottles to WGL.

Cooler (field) blanks:

(1) Plastic bags fit to hold one container will be purchased by WGL
(2) Cleaned bottles will be filled with reverse osmosis tap water at WGL and autoclaved.
(3) After cooling, bottles will be securely closed, and placed in the bag.
(4) Bags will be twisted closed with wire closures.
(5) Filled bottles will be distributed at the annual field training meeting held at WGL so that each field station will start out the season with the total estimated number of field blanks needed for the sampling season.
(6) Field stations can store these prepared field blanks as long as needed, because as each bottle is wrapped individually.
(7) Field stations will prepare coolers according to the QAPP, in as clean of an area as possible at the station.
    a. Anytime boxes are opened, staff should be in clean clothes and use a new pair of clean gloves for actually handling the bottles. For example, you may open the box with un-gloved hands. Then, put on gloves to open the bag and touch the bottles.
(8) Once clean coolers are ready, boxes may be opened, bottles labeled, and placed into clean coolers.
(9) After use, field staff can use the same bag and box to return the bottles to WGL.

WGL will have designated shelves in the garage for boxes of dirty bottles.
A note on labels: We've found some great labels that withstand all sorts of abuse and are available on GSA. Avery laser white weatherproof address labels #5520.

Appendix E

Methods for Elimination and Reduction of eDNA on Boats and Equipment

Recommended methods for reduction of residual or environmental DNA on boats and other equipment associated with environmental conservation work in the field. For boats and equipment that have been previously exposed to carp DNA, choose one high pressure sprayer method ***in conjunction*** with one chemical method to reduce DNA levels to negligible or below LOD. Read MSDS and Use personal protective equipment (PPE). Read job hazard assessment (JHA) for applicable methods.

Method	Active Ingredient	Contact Time	Advantages	Disadvantages	Cautions
Steam + Pressure Washer @ 212 F	Pressure, Heat and Water	10 sec	Environmental safety	Need electrical and water hookups Can melt or tear materials	Use PPE – need proper safety training, can cause burns, cuts, air embolisms
Cold Water High Pressure Sprayer with Low Pressure Detergent Application	Detergent, Pressure and Water	3-5 min Detergent contact/ 10 sec high pressure rinse	Environmental safety	Need water hookups Can tear materials	Use PPE – need proper safety training, can cause cuts, air embolisms
10 % Household Bleach Low Pressure Saturation	Sodium hypochlorite (5-8% before mixing)	10 min	No hookups necessary – can use off station	Not preferred for environmental safety Single day use in solution	Use PPE and avoid breathing fumes
20 % Household Bleach Immersion Bath	Sodium hypochlorite (5-8% before mixing)	10 sec	No hookups necessary – can use off station	Not preferred for environmental safety Single day use in solution	Use PPE and avoid breathing fumes
2% Virkon Immersion Bath	Potassium Peroxymonosulfate and Sodium Chloride	30 min	Lasts 1 week in solution Environmental safety	Corrosive to metals when exposed longer than 10 min	Use PPE – wear a dust mask when mixing powder
2% Virkon Low Pressure Saturation	Potassium Peroxymonosulfate and Sodium Chloride	10 min	Lasts 1 week in solution Environmental safety	Not quite as effective as bleach in laboratory study; equal or better than bleach in field (boat) equipment study	Use PPE – wear a dust mask when mixing powder. Do Not Aerosolize! Use a low pressure dispenser (hose attachment sprayer at largest droplet setting)

Preventing the Spread of Pathogens, Bacteria and Invasives on/in Boats, Motors, Trailers and Equipment

Developed by the U.S. Fish and Wildlife Service Midwest Region

Disinfection Techniques and Options:

Boat, motor, trailer, and gear must have all aquatic vegetation, visible organisms/animals, soil, and water drained and removed BEFORE TRANSPORT. Upon leaving a water-body possibly infected with pathogens or invasive species, a proper disinfection must be completed before re-use of boat, motor, trailer, and any exposed gear in another waterway. Contact time is crucial for complete disinfection. Contact time reflects exposure of air, water, or disinfectant to a specific area, and not the total amount of time spent disinfecting. For example, if you are using 50C water to disinfect your boat, you must apply 50C water to each area for ten minutes or longer (see options and procedures below). Read MSDS, wear personal protective gear (ppg), and comply with federal and state regulations.

Disinfection is MANDATORY for all exposed equipment and gear!

Methods	Procedures	Positives	Negatives
Heat + Air (Drying in hot sun/air)	**30C (86 F) 24 hours minimum** (time at temp contact period crucial) (exposure to hot sun/ air while dry)	Chemical free Effective, but only if properly done under ideal conditions	Time consuming Weather/Temperature criteria critical to reliable results.
Heat + Water Spray &/or Immerse	**50C (122 F) contact time 10 minutes** (time and temp contact crucial) (source of very hot water needed)	Chemical free Same as above	Must maintain high water temp/contact; hotter than normal tap or carwash. Use personal protective gear (ppg).
Steam Spray *(approved for eDNA decontamination)	**100C (212 F) contact time 10 seconds** (time and temp contact crucial) (steamer washer/ sprayer needed)	Chemical free Same as above	Must maintain very high water temp/contact; (i.e. steamer washer/sprayer). Risk of burns use ppg.
Virkon Aquatic *(approved for eDNA decontamination in a 2% solution for 10 - 30 min)	**Follow product directions for proper mixture and minimum contact time** (apply directly, maintain saturation and rinse thoroughly)	Environmentally friendly Designed for aquatic use Quick inactivation time Sewer compatible	Follow MSDS directions for health risks and use ppg when mixing. Chemical based. Corrosive in concentrate form.
Quaternary Ammonium + Water (family of products)	**Follow product directions for proper mixture and minimum contact time** (apply directly, maintain saturation and rinse thoroughly)	Effective, user friendly Low health risks Sewer compatible	Follow MSDS directions for health risks and use ppg. Chemical based.
Sodium Hypochlorite + Water *(approved for eDNA decontamination)	**200 ppm for pathogens; 5000 ppm for eDNA - contact time: 10 minutes** (apply directly, maintain saturation and rinse thoroughly)	Widely available Effective	Follow MSDS directions for health risks and use ppg. Highly Corrosive.

USFWS Contacts: Corey Puzach, Fish Health Center: corey_puzach@fws.gov, Dave Wedan, Watercraft Safety Coordinator: dave_wedan@fws.gov

*for eDNA decontamination details contact Jen Bailey, Whitney Genetics Lab: jennifer_bailey@fws.gov

updated: April 2014

Appendix F

Mobile eDNA Trailer Maintenance and Use Manual

Mobile eDNA Trailer Maintenance and Use Manual

Purpose

In order to process water samples collected in the field, a sterile work environment is required to filter/centrifuge water while preventing any field contamination. The eDNA trailer is designed to be mobile while maintaining the highest level of quality assurance and quality control that would be found in any lab environment.

This is done in part by keeping all dry consumable equipment (filters, paper towel, gloves) in locked cabinets within the dry lab area. All equipment that requires decontamination between uses (filter funnels, carboys, filter manifolds) are stored under the work bench in the wet lab.

The eDNA trailer is equipped with two sinks, both of which are in the wet lab. The aft sink should be considered contaminated and the forward sink should be considered clean (non-contaminated). The forward sink has two faucets; one for deionized (DI) water and one for regular tap water.

Placement of the eDNA Trailer is important in optimizing its capabilities and improving the efficiency of sample processing. The eDNA trailer should be set up near an access point to the body of water being sampled, preferably upriver of where collections begin.

Cautions:

- Park eDNA trailer on level hard surface to prevent getting stuck and to keep filtering/centrifuging equipment functioning properly.
- Park eDNA trailer so exhaust from generator is blown away from eDNA trailer (if possible).
- If eDNA trailer is equipped with a dry ice cooler, turn on ceiling fan in dry lab to remove any carbon dioxide gas once trailer is set up and running,.
- When the ultra-violet (UV) system is in operation, no staff should be in the wet lab.

If eDNA trailer will be set up at the same place for an extended period of time, disconnect from tow vehicle. If eDNA trailer will be located at a particular site for the day, it is acceptable to leave it connected to tow vehicle provided staff still go through the leveling procedures (see set up procedures section).

Tools Needed:

- Leveling jack socket
- Level
- Wheel chocks
- Four blocks of wood (2x4-12” long) to be placed under leveling jacks.

eDNA Trailer Set Up Procedure

1. Park eDNA trailer on flat hard surface. If using external water source, ensure there is enough water hose to reach water connection.
2. Place wheel chocks in front of and behind trailer tires.

3. Leveling the eDNA trailer.
    a. Use the power jack on tongue of eDNA trailer to visually level trailer.

b. Using a leveling jack socket, lower the four leveling jacks to the ground. Place blocks of wood under leveling jack pads. (leveling jacks are located at the four corners of the trailer).
c. Enter the eDNA trailer and place level on wet lab counter top to identify if eDNA trailer needs any further leveling (accomplished by putting weight down with leveling jacks located on all four corners of the trailer).
d. The four leveling jacks are used for making fine adjustments. The bulk of the leveling procedure should be accomplished with the power jack located on the tongue of the eDNA trailer.

4. Connecting power source (ensure all operating system switches are in the off position).
    a. Operating from shore power.
        i. Make sure to use 220v, with a 30 amp minimum service, but it cannot exceed a 50 amp service. If the proper shore power requirements are not available use on-board generator.
        ii. First connect the extension cord to the eDNA trailer, then plug into the power source.
        iii. Once the shore power is properly connect, switches for operating systems can be turned on.
    b. Operating generator (ensure all operating system switches are in the off position).
        i. To start generator, hold down generator starter in the priming position for 30-60 seconds.
        ii. Push generator starter switch to the on position until generator starts.
        iii. Once the generator is running, switches for the operating systems can be turned on.

5. Connecting water source (fill internal water tank before heading into field).
    a. If using external water source, connect hose to eDNA trailer and water source. Once the connection is made, turn on the water source to pressurize the water line to the trailer.
    b. If using internal water source, turn on water pump.

Operating Systems

1. Supplies
    a. All consumables (gloves, filters, paper towels) are stored in the dry lab.
    b. All filtering equipment is stored in wet lab in side cabinets.
        i. Filter manifolds, carboys, forceps, tubing, funnels are to be kept in the wet lab at all times.

2. Lighting
    a. Light switches for wet lab are located in wet lab.
    b. The ultra violet light (UV) on/off switch is located in dry lab with a flip-up plastic lid to prevent accidental use.
        i. When UV system is on, a warning indicator light in wet lab will alert anyone working in wet lab that UV system is in on.

3. HVAC
    a. Heating/ Cooling: Adjust thermostat to desired temperature for working conditions
    b. Dry Lab Ceiling Fan: Set thermostat to turn on the fan.

CAUTION: DRY LAB CEILING FAN SHOULD ALWAYS BE ON WHEN DRY ICE IS KEPT IN DRY ICE COOLER.

4. Vacuum System

CAUTION: DO NOT LET VACUUM PUMP RUN MORE THAN 60 SECONDS WITHOUT an OPEN VALVE.

    a. Vacuum pumps are located in a cabinet in dry lab.
    b. To turn on vacuum pump, locate the switch in the wet lab and move to "on" position.
        i. Each pump is set to operate each filter line on each side of trailer.
        ii. Two shutoff valves located next to forward sink controls which vacuum lines are activated.
        iii. Once filtering is complete, open several valves and allow the vacuum pump to operate without pressure for at least 5 minutes before shutting off.

5. DI/water heater (if using internal water source turn on water pump).
    a. Water heater
        i. On/off switch located in dry lab next to thermostat.
        ii. Water heater should be turned on once filtering begins to ensure water is hot.
        iii. When red indicator light turns off water is hot.
        iv. Front sink left handle produces hot water.
    b. DI system
        i. Front sink right handle produces DI water.
        ii. DI water should be tested using TDS meter to ensure water quality is met.
        iii. When DI water TDS is above 15ppm change resin in tank 1 and tank 2.
6. Refrigerator
    a. Runs off propane/electric

Laboratory Decontamination

After trailer has been leveled and set up, decontaminate the interior of the trailer at the beginning of each day:

1. Mix up a 10% bleach solution in bleach bath container.
2. Wipe down all counters and cabinets with bleach solution.
3. Wipe down all counters and cabinets with tap water to remove bleach residue.
4. Sweep the floor to remove any debris brought in on boots or coolers.
5. Mop floor with 10% bleach solution and rinse with water to remove any bleach residue.
6. Lock back door and dividing door of the wet/dry lab. Turn on the UV lights for 30 minutes.
7. After UV lights have been turned off, unlock trailer doors and begin setting up the wet lab for filtering/centrifuging.

Filtering/ Centrifuging Procedures
See QAPP 2014

eDNA Trailer Shutdown Procedure
Centrifuging:

1. Store centrifuging equipment in curb side cabinets.
2. Wipe out centrifuges with dry paper towel.
3. Secure centrifuges for travelling.

Filtering:

1. Once the last sample is filtered, disconnect all carboys from vacuum line. Completely open four or more valves and let vacuum pump run for 5 or more minutes. Doing so will remove any moisture in vacuum line, preventing long term damage to vacuum pump.
2. Store filtering equipment in curb side cabinets.

Towing Preparations

1. Remove garbage from inside eDNA trailer and place in nearest receptacle.
2. Thoroughly wipe down all countertops with water. (Any bleach residue will discolor and rust counter top).
3. Shut down all operating systems.
    a. Turn off
        i. Vacuum pumps
        ii. Lighting
        iii. Water pump
        iv. Water heater
        v. HVAC/ refrigerator
        vi. Ceiling fan (make sure roof vent is closed)
4. Disconnect water source.
5. Disconnect power source.
    a. If running generator, allow generator to run without load for 5 minutes before shutting down.
6. Raise leveling jacks
7. Connect eDNA trailer to truck (if needed).
    a. Connect/check safety chains.
    b. Raise power jack high enough to avoid rubbing ground.
    c. Connect/check trailer lights and ensure they are functioning properly.
8. Remove tire chocks.
9. Take final walk in and around eDNA trailer.
    a. Ensure eDNA trailer is ready for traveling.
        i. Secure all equipment
        ii. Close all windows and cabinet doors.
        iii. Lock all doors including lab dividing door.

iv. Put caps on holding tanks.
v. Raise steps.

Trailer Maintenance

1. Before departing on each trip check tire pressure and tread wear.
2. Check hour meter on generator and service as prescribed in owner's manual.
3. Check propane cylinders to ensure they are still properly secured and inspect propane lines for cracking.
4. Ensure DI system is working properly, if necessary, replace resin.
5. Periodically check MSDS sheets, med kits, and fire extinguishers to ensure they are all update
    a. One safety item to keep in the trailer is kitty litter to place on any diesel spilled on the ground.

Appendix G

eDNA Security Plan for Co-Located La Crosse FWCO and WGL

Standard Operating Procedure for Minimizing Risk of Invasive Carp DNA Contamination within the Midwest Fisheries Center

eDNA Security Team: Nicholas Bloomfield, Ken Phillips, Jen Bailey, Mark Steingraeber
Project Leaders: Scott Yess, Terrence Ott, Emy Monroe
Safety Officers: Mark Steingraeber, Nikolas Grueneis, Terrence Ott
Effective Date: April 20, 2014 Review Date (annually): April 2015

Purpose: The shared location of three U.S. Fish and Wildlife Service (USFWS) Offices, La Crosse Fish and Wildlife Conservation Office (LFWCO), La Crosse Fish Health Center (LFHC), and Whitney Genetics Laboratory (WGL) presents a unique challenge to the USFWS Environmental DNA (eDNA) Monitoring Program. eDNA sample processing is a sensitive process, inherently susceptible to contamination from outside sources. Processing of eDNA samples requires strict adherence to laboratory hygiene and specific protocols for reduction of risk factors that could cause contamination of samples. Sample analysis for the entire eDNA monitoring program, currently including samples from the Great Lakes and Mississippi River basins, is performed at the Whitney Genetics Laboratory. Laboratory staff minimizes risk by following procedures designed to reduce introduction of DNA to the laboratory that may be on surfaces of sample packages, sample bottles, clothing and footwear. To compound risk factors, a mission of the co-located LFWCO is to monitor invasive carp populations directly, which includes collection of invasive carp and invasive carp tissue samples, as well as collecting water samples for eDNA analysis at WGL. Cross-contamination between eDNA samples and gear and carp sampling gear could be very likely if protocols were not in place to help staff address contamination challenges. The co-located La Crosse Fish Health Center occasionally receives whole carps and/or carp tissue samples for diagnostic fish health cases, and maintains several invasive carp tissue cell lines for research purposes as part of its mission. Midwest Fisheries Center staff, collaborators, volunteers, and associated sample containers, laboratory and field equipment have a large potential for acting as vectors of contamination by carrying invasive carp DNA to and within the co-located field station and indirectly to WGL. Due to the unique challenges that co-location of these offices presents, stringent protocols must be followed to prevent contamination of water samples during collection, sample receipt, movement through the building and shared spaces, and sample processing. Following these isolation and decontamination protocols will reduce unintentional transfer of invasive carp DNA to facilities, sampling gear, eDNA water samples and sample containers, field and lab personnel, and other surfaces.

Scope: This document identifies high risk zones for movement of personnel, collaborators, volunteers, visitors and fish or tissue samples throughout the facility, sets up communication practices for personnel to help contain known contamination risks so that they may be isolated and eliminated, and provides directions for the isolation and decontamination of field and laboratory equipment, samples, and packages.

Responsibilities: USFWS employees, collaborators and volunteers are responsible for following procedures outlined in this document and reviewing updates to this document annually. Visitors will be notified of procedures applying to the specific activities of their visit.

Definitions:
Invasive carp = silver and bighead carp.
Established invasive carp populations = waters where silver carp are observed jumping, or a commercial harvest is occurring.

Risk Zones: The facility is divided into zones of contamination risk for personnel or equipment dependent on the activities that may take place within (Figure 1).

- Zone A is the area of highest contamination risk. This area includes the gated outdoor area directly in front of the three south garage bays bordered by fences to the south and east, and a path from the entry gate to this area. Direct contact boats, vehicles, sampling gear, and nets may be stored in this area. This zone will also serve as the decontamination area for all traditional sampling gear and equipment.
- Zone B is the area with a moderate or periodic risk of contamination present. Areas with continuous moderate risk include the virology laboratory, the three LFWCO garage bays, the fisheries laboratory, and the woodshop. Invasive carp tissue cells are cultured in the virology laboratory and present a continuous risk. Aseptic techniques are applied to methods in all tissue cell culture procedures, which will minimize risk for escape of DNA from this laboratory. In addition to these practices, 10% bleach or DNA away will be applied as needed to equipment and laboratory surfaces when working with invasive carp tissue cells. Predominately decontaminated equipment and gear will be housed in the garage area, fisheries laboratory, and the woodshop, although in special circumstances direct contact gear, direct contact equipment, and invasive carp or their parts may be present. Specific protocols are in place to reduce contamination threats from these sources. Areas with periodic risk of contamination are the front door and reception area where fish and/or tissue samples may arrive for fish health diagnostic cases or research purposes. In this case diagnosticians will remove sample packages to the LFHC histology laboratory for processing. Specific protocols are in place to contain contamination threats and to communicate risks to building staff. In the rare case that a partner or member of the public in possession of an invasive carp brings carcass(es) into the building through the front entrance for reporting, Center staff will contain the risk and notify building personnel of the specific contamination potential associated with each individual case.
- Zone C is the area with a low contamination risk. This area includes the interior of the LFWCO, LFHC, the office and hallway space of WGL, the north garage bay, and the remainder of the gated parking lot aside from Zone A. Predominately only decontaminated personnel, equipment, and gear will be housed in this zone. Under special circumstances, contaminated data sheets, personnel, and invasive carp or their parts may be present. Specific protocols are in place to reduce contamination threats from these sources.
- Zone D is the area where there is a very small risk of outside contamination. This area includes the laboratories of WGL. Personnel and equipment entering Zone D must adhere to strict protocols prior to and upon entry or exit to reduce the potential of outside contamination into the laboratory.

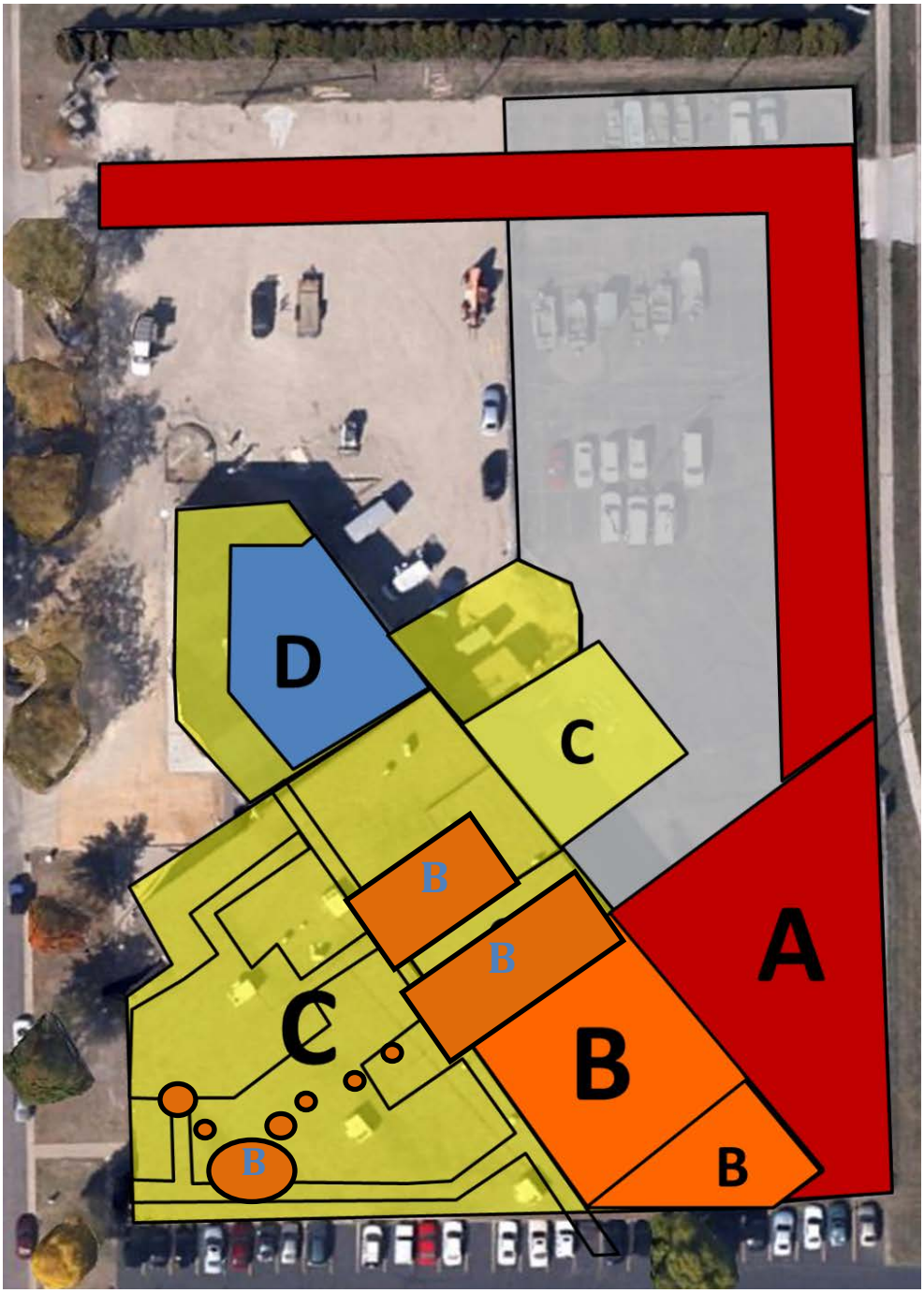


Figure 1. Zone map of the Midwest Fisheries Center Facility. Zones indicate the likelihood of invasive carp DNA being present, from A (red) the highest to D (blue) the lowest.

Movement of samples, equipment and people: Each office has outlined specific procedures to minimize contamination threats for tasks that occur within respective programs and may not carry over to the other co-located offices (see procedure details below). However, many times tasks and group projects are shared or overlap between offices, so staff, volunteers, and collaborators must have procedures in place that encompass a wide variety of tasks and projects that fall into gray or shared

areas. All participants must be aware of decontamination or risk minimization procedures that apply to daily duties as well as shared or overlapping duties. All staff, collaborators and volunteers are responsible for knowing Risk Zones and procedures that apply to entering each risk zone. Staff is also responsible for making collaborators, volunteers and visitors aware of procedures that apply to them and their corresponding activities. If staff or volunteers from one office will be working in the workspace of another office, or collaborating with another office, they must be familiar with SOP's pertaining to the work they are performing. Samples and equipment must be handled according to protocols, no matter who receives samples or packages, or which staff member is using the equipment. Much of the equipment in use for the Center is shared. Be aware of procedures to follow when borrowing or using shared equipment.

Decontamination Gear and PPE: There will be two separate decontamination zones, with dedicated equipment to remain in each zone. One zone will be for medium to low risk gear, and one for high risk gear. Clean sets of personal protective equipment (PPE) will be stored in cabinets in each zone (or nearby) that include boot covers, Tyvek suits, lab coats, and decontamination chemicals (Virkon, etc). This PPE should be worn while decontaminating gear, and then removed before reentry into low risk areas. In Zone C of the garage, a "clean" cold water pressure washer with attachments for degreasing detergent will be installed for use by Center staff to decontaminate medium to low risk field equipment and gear such as eDNA boats and equipment and boats and equipment used in Wild Fish Health Survey sample collections where invasive carp populations are not established. A duplicate set of decontamination equipment and PPE for use with highly contaminated boats, equipment, etc will be kept near the hot Zone A. Do not use this equipment for low-contamination equipment. This equipment and associated PPE will stay in Zone A. Keep "clean" and "dirty" equipment separate and do not move outside of their appropriate Risk Zones. Boot covers and Tyvek suits will be kept at entrances and transition areas for use as needed to prevent transfer of DNA on surfaces, boots, and employees.

Communications: Specific communications protocols for certain tasks are outlined in SOP's (below), but many communications are essential for maintaining the integrity of Risk Zones, samples, packages, and shared spaces. Please inform Center staff of risks they may become involved in to make sure they are aware and follow appropriate procedures to minimize risk. Inform Project Leaders and the eDNA and Biosecurity Team members (Nicholas Bloomfield, Ken Phillips, Jen Bailey, Mark Steingraeber) of known or unusual risks that are not covered in Procedures listed below. Inform staff members who will be working with equipment, samples, or in areas that may become affected of any procedures they may follow to reduce DNA contamination and prevent spread throughout the building. If this may also include a safety risk, inform Safety Officers (Nick Grueneis, Mark Steingraeber, Terry Ott) as well.

La Crosse Fish Health Center Standard Operating Procedures: The La Crosse Fish Health Center (FHC) follows guidelines established in the American Fisheries Society Fish Health Section Bluebook, Section 3: Quality Assurance/Quality Control, which establishes procedures for handling samples. The section provides basic guidance to prevent contamination in the laboratory and between samples.

Although not typical, the La Crosse FHC occasionally receives whole invasive carps and/or tissue samples from invasive carps as diagnostic cases. The La Crosse FHC also maintains several invasive carp tissue cell lines for research purposes. To minimize the spread with in the Midwest Fisheries Center building of DNA from invasive carps, the La Crosse FHC has adopted additional procedures for handling invasive

carps, tissue samples from invasive carps, and invasive carp tissue cultures. Procedures to be followed in addition to those described in Section 3 of the AFS-FHS Bluebook are:

1. La Crosse FHC staff will provide notification to Whitney Genetics laboratory staff that invasive carps and/or tissues are being worked with. WGL staff will take appropriate precautions.
2. Any whole invasive carps or tissue samples from invasive carps received at the La Crosse FHC will be in a sealed container. The external surface of the container will be coated with an appropriate decontaminant (10 % bleach or DNA away) for a minimum of 10 minutes prior to opening. Isopropanol and quaternary ammonium products (Lysol Professional, Extra) are not acceptable decontaminants for eliminating DNA).
3. Whole invasive carps will be dissected/processed within the Histology Laboratory (Room 10) at the La Crosse FHC. Processed tissue samples will be distributed to the appropriate laboratory(s) for pathogen screening.
4. La Crosse FHC staff will don appropriate PPE when working with invasive carps, invasive carp tissues, or invasive carp tissue cell culture. Appropriate PPE will be rain gear, Tyvek, or other designated clothing, and boots when dissecting invasive carps or processing tissues from invasive carps. Laboratory coats are considered minimum PPE and are only allowed for tissue cell culture work, or other laboratory work involving processed samples (histopathology samples, bacterial isolates, etc.). Laboratory coats and other PPE that may be contaminated with invasive carp DNA should not leave the laboratory until they have been washed, decontaminated or disposed of.
5. Immediately following completion of work involving invasive carps or their tissues, La Crosse FHC staff shall treat all laboratory surfaces, equipment and tools with an appropriate decontaminant (10 % bleach or DNA away) for a minimum of 10 minutes.
6. PPE shall be removed and placed in appropriate locations for disposal or cleaning. Employees shall thoroughly wash their hands with soap and water.
7. Any La Crosse FHC employee that worked directly with invasive carps and/or tissues from invasive carps, or may have potentially been exposed to invasive carp DNA, shall not enter Zone D until the following day.

La Crosse Fish and Wildlife Conservation Office Standard Operating Procedures: The following procedures are specific to La Crosse Fish and Wildlife Conservation Office programs and projects.

Separation of Field Equipment: To reduce the threat of contamination, field crews will maintain two separate sets of personal field sampling gear including at a minimum PFD's and rain gear: 1) eDNA sampling gear and 2) Any other traditional field applications. Equipment used for eDNA sampling versus traditional sampling will also be maintained separately: 1) One vehicle and one boat with the necessary associated equipment (oars, fuel tanks, tool box, etc.) will be dedicated to eDNA sampling and 2) Remaining boats, vehicles, and other gear will be designated for any other use. All eDNA field equipment (boat, vehicle, PFD's, rain gear, etc.) and supplies (gloves, filters, centrifuge tubes, etc.) will be housed at a separate storage location. Contaminated traditional sampling equipment will be predominately staged or stored in Zone A. Equipment that has been decontaminated and decontamination supplies will be housed in Zone B.

Decontamination Equipment List:

This decontamination equipment will be maintained separately between eDNA sampling and traditional sampling

- Brushes
- Water pump and/or buckets
- DNA Away or equivalent
- Paper Towels
- Garden Hose
- Garden Sprayer
- Pressure washer with steam option
- Virkon Aquatic
- >30 gallon decontamination tubs
- Personal Protective Equipment (PPE)

Decontamination Procedures:

Procedure A- Decontamination of eDNA sampling gear and equipment: This procedure refers to actions taken prior to leaving and upon returning to the station for collection of eDNA samples in the field. Equipment designated for eDNA gear and equipment decontamination will be used to perform any functions related to eDNA sampling. This equipment will be housed in Zone C of the garage. This procedure does not preclude additional protocols outlined in the Quality Assurance Project Plan (QAPP) performed prior to eDNA sampling. Due to the sensitivity of eDNA sampling, this must be done before and after each eDNA sampling trip.

1. Prior to leaving on a collection trip, use DNA Away or equivalent to decontaminate any personal items that will be used on the trip (watches, sunglasses, etc.)
2. Park truck and boat in Zone C.
3. Use pressure washer to rinse truck (including floor mats), boat, and any associated equipment capable of withstanding a high pressure rinse (oars, fuel tanks, tool box, totes, etc.). This could include PFD's, rain gear, or boots if they were heavily soiled.
4. Fill a pump sprayer with 2% Virkon Aquatic.
5. Spray all surfaces of truck, boat, and other equipment until all surfaces are completely soaked. These surfaces must be coated for at least 20 minutes. Rinse when complete.
6. Mix a batch of 2% Virkon Aquatic according to recommendations in the decontamination tub.
7. Dip PFD's, rain gear, boots, floor mats, oars, or any equipment capable of withstanding a prolonged submergence without damage. Allow to soak for 20 minutes.
8. Allow equipment to air dry on the designated drying racks prior to returning all eDNA equipment to the off-site facility.

Procedure B- Minimize Invasive Carp Residue Transport to Station: This procedure refers to returning from sampling that has occurred in waters with any reported invasive carp observations.

1. Prior to returning to the station, use water pump, buckets, and brushes to remove as much blood, slime, scales, sediments, etc. from the boat, equipment, and gear as possible.

Procedure C- Storage or Decontamination of Traditional Sampling Gear and Equipment: This procedure refers to returning from sampling that has occurred in water with any observed invasive carp.

Equipment designated for traditional sampling gear and equipment decontamination will be used to perform any functions. This equipment will be housed in Zone B of the garage. Boats, vehicles, gear and non-enclosed equipment that will be stored inside the facility will be decontaminated. Note: Gear and equipment from non-invasive carp infested waters are still subject to disinfection standards prior to entering a different body of water.

1. Park vehicle and boat in Zone A. These and any gear or equipment may remain in Zone A without decontamination.
2. Personnel wearing direct contact clothes or shoes/boots should not proceed beyond Zone B.
3. Gill/trammel nets will be laid out to dry within Zone A. After drying, these will be stored in Zone A or enclosed with lids if storing in Zone B. Prior to entry to Zone B, the outside of the container will be decontaminated in step 6 of this procedure. Fyke/mini-fyke nets will be decontaminated in step 6 of this procedure prior to storage in Zone B.
4. Use hot pressure washer to rinse truck (including floor mats), boat, and any associated equipment capable of withstanding a high pressure steam rinse (oars, fuel tanks, tool box, totes, etc.). This could include PFD's, rain gear, or boots if they were heavily soiled.
5. Mix a batch of 2% Virkon Aquatic according to recommendations in the decontamination tub.
6. Dip PFD's, rain gear, boots, floor mats, oars, or any equipment capable of withstanding a prolonged submergence without damage. Allow to soak for 20 minutes. Rinse when complete.
7. Allow equipment to air dry on the designated drying racks.
8. Decontaminate cleaning supplies in tub and replace in Zone B
9. Data sheets and clipboards will be kept in an enclosed container within FWCO offices when not in use or in the FWCO lab. After handling sheets for data entry or verification, replace into enclosed container, clean work area with DNA Away or equivalent, and wash hands.

Procedure D- Net Mending: This procedure refers to mending gill nets, trammel nets, and fyke/mini-fyke nets that have been used in waters with any known invasive carp observations.

1. Prior to handling nets, personnel will don appropriate PPE (rain gear, Tyvex, or designated clothing and boots).
2. When possible, mending will take place in Zone A. When necessary, mending will take place within the woodshop section of Zone B. When working in Zone B, area will be sectioned off and labeled as a DNA hot zone.
3. Nets will be decontaminated via Procedure C-8 in the southern garage section of Zone B.
4. Prior to entering Zone C, any PPE will be removed and stored within the labeled hot zone and hands will be washed.
5. Upon completion of net mending, PPE and net mending tools will be decontaminated via Procedure C-8.
6. Remove any solids (leaves, scales, algae, etc.) that have accumulated on the floor.
7. Prepare a solution of decontaminate and mop floor.

Procedure E- Reduce Transport of DNA Contamination between Zones: This procedure refers to movement of personnel, gear, and equipment throughout the facility. Specific protocols must be followed to reduce the likelihood of spreading invasive carp DNA into zones of lower risk and ultimately WGL.

1. Personnel working with direct contact contaminated gear in Zone A or B will wear appropriate PPE. PPE will be removed and hands washed prior to re-entry into Zone C.
2. Personnel entering Zone D from Zone C will at minimum wash hands prior to entry.

3. It is recommended that personnel working in Zone D do not enter Zone A or B. If it is necessary, they will wear PPE to cover footwear and clothing prior to entering Zone A or B and remove upon exit.
4. Any potentially contaminated gear will be decontaminated via procedures above or enclosed prior to entry to Zone C or D.

Whitney Genetics Laboratory Standard Operating Procedures: Most of the procedures performed at Whitney Genetics Laboratory are specifically for eDNA monitoring of invasive carps and are incorporated into the Quality Assurance Project Plan (QAPP). Occasionally, duties are performed either in addition to or in support of the QAPP, or for programs not related to the QAPP such as flow cytometric analysis of grass and black carp samples. Samples are also occasionally processed at Whitney Genetics Laboratory in support of research and development of the QAPP, or for other USFWS programs. The following procedures will be followed in addition to QAPP procedures while in support of the eDNA program, performing research, or during development of new programs.

1. Any staff, collaborators, volunteers, or visitors to the WGL who are wearing clothing or footwear that has been exposed to any water where invasive carp are known to exist must wear Tyvek suits, lab coats, gloves or boot covers as appropriate before entering the lab. These are provided at the two main entrances at WGL. Any WGL staff or associated member who is leaving the laboratory for an area of known contamination should bring PPE with them to minimize risk of becoming contaminated while outside the lab. Dispose of contaminated gear before re-entry to the lab. Change footwear if possible.
2. When receiving samples, equipment, or packages that may be contaminated with invasive carp DNA, wear appropriate PPE to help contain contamination and dispose of PPE, gloves, and boot covers after each use. Stay in receiving room and don't remove samples or associated equipment to another room until all surfaces have been decontaminated.
3. Spray surfaces of containers or equipment with 10% bleach (10 min exposure) or DNA Away and wipe surfaces with paper towels. Remove to storage or to work area within the receiving room for processing.
4. When working with tissue samples from grass carp or black carp (i.e., not silver carp or bighead carp) use bench paper to define working space and keep associated laboratory instruments, etc, confined to workspace. After task is complete, decontaminate instruments with 10% bleach (10 min) or DNA Away and wipe entire area with either decontaminant. Contain contaminated waste products in a sealed waste bag and remove to trash bin.
5. Decontaminate surfaces of any sample containers saved for storage in WGL before removing to storage or other sample processing rooms.
6. Direct work with invasive carp tissue cells is important for preparation of extraction control samples for the eDNA monitoring program and sometimes for other purposes. If at all possible, perform this work in borrowed laboratory space from LFHC or another appropriate space. Wear protective gear to contain DNA contamination during sample processing, use 10% bleach or DNA Away before, during, and after processing procedures, and dispose of contaminated PPE. Follow all procedures outlined for the laboratory space you are working in.

In addition to step-by-step procedures, it is pertinent for all WGL employees to follow immaculate laboratory hygiene practices:

- Dedicate footwear to laboratory space. This footwear should not be worn in Risk Zones A or B and should be worn only in Risk Zone D if possible. If you must enter Risk Zone C during times of known contamination, wear boot covers while working in that area and dispose of later.
- Clothing or attire should not have been exposed to any potential contamination risks without laundering before entering WGL. This applies to hats, jackets, boots, shoes and regular clothing. Any attire that has been worn in areas of potential risk must be laundered using detergent or decontaminated with DNA Away before returning to the lab. If you have been sampling for the

eDNA program, volunteering with other programs, recreating in areas of known contamination, or performing research duties do not return to the lab wearing the same clothes.

- Wash hands with copious amounts of soap and water if you have been exposed to invasive carp DNA.
- Be aware of any potential risks that may not be covered in these protocols and inform teammates of any concerns.